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83. (New) The chimeric peptide-nucleic acid fragment according to claim 5, wherein the nucleic acid hybridizes to form a linear single-strand structure with an overhanging 5' end.

REMARKS

REVISED FIGURES 5B AND 5C

Applicants herewith submit revised figures 5B and 5C. The last column of the nucleotide sequences of these figures contain only 9 residues. The corresponding figures in the parent PCT application correctly contain 10 residues. Applicant herewith respectfully requests entry of the corrected figures. No new matter is believed introduced by way of this entry.

AMENDMENTS TO THE CLAIMS

The claims have been amended to correct a number of grammatical deficiencies pointed out by the Examiner and to more clearly define what the Applicants consider the invention. The amendments do not introduce new matter, and they are fully supported by the specification and the claims as originally filed. Therefore. Entry under 37 C.F.R. § 1.111 is respectfully requested,.

SUBSTITUTE SPECIFICATION

Applicants herewith resubmit the substitute specification in compliance with 37 C.R.R. 1.125(b), along with 1) a statement that the substitute specification contains no new matter; and 2) a marked-up copy showing the amendments to be made via the substitute specification relative to the translation of the German version of the patent application as originally filed. In the light of the Examiner's comments concerning the substitute specification filed December 16, 1996, Applicants have corrected the spelling and grammatical errors therein and deleted the Table of Contents. Applicants bring to the Examiner's attention the fact that the parent PCT application originally filed (in German) did not comprise a "Summary of the Invention". Applicants have therefore copied the Abstract of the substitute specification into the section entitled "Summary of the Invention" in the

substitute specification attached herewith. No new matter has been introduced by way of this substitute specification. Applicants respectfully request the Examiner's consideration and entry of the substitute specification attached herewith, in place of the substitute specification filed December 16, 1996.

Applicants have, throughout the substitute specification, chosen to replace every occurrence of the term "chimerical" with "chimeric". This change brings the terminology in line with usage prevalent in the art and does not introduce any new matter. With this amendment, Applicants have also introduced a similar change througout all of the pending claims.

OBJECTION TO THE DRAWINGS

Applicants thank the Examiner for pointing out the German axis label in Figure 6B. Applicants will submit a corrected Figure 6B at the time that all of the formal drawings are submitted.

OBJECTIONS TO THE CLAIMS

Applicants have, with this amendment, brought claims 18 and 35 into proper dependent form. Claim 18 now recites a signal peptide which carries a "cell-specific, compartment-specific or membrane-specific recognition sequence." Support for this amendment may be found at pages 10 and 12 of the substitute specification filed herewith. Claim 35 has been amended to limit the term "mitochondrial transcription termination factor" to one that is "bidirectional", thereby further limiting Claim 34. Support for this amendment to Claim 35 can be found on page 17 of the substitute specification filed herewith.

Applicants have amended occurrences of the term "grouping(s)" in Claims 10, 11, 12, 13, 17 and 22 to "group(s)". A subsequent amendment to Claim 22 renders this objection moot, however.

Applicants have inserted the word "to" into Claim 16.

Applicants have deleted the phrase "any one of" from Claim 21, thereby bringing it into proper dependent form.

Applicants have replaced the word "liked" by "linked" in Claim 25.

Applicants have deleted the term "(phosphorylated)" in Claim 44 and added new dependent claim 62 which specifies a further limitation that the ends of the nucleic acid

fragment are phosphorylated. Support for this amendment is found on page 19 of the substitute specification.

Applicants have inserted in Claim 61 an additional bracket to pair with the "extra" bracket pointed out by the Examiner. Applicant respectfully submits that it should now be clear that the word "employing" is to be deleted from the Claim.

REJECTIONS OF THE CLAIMS

Rejections Under 35 U.S.C. § 112, Second Paragraph

The Examiner has rejected Claims 16, 17, 21, 26, 27, 29, 36, 39, 52 and 58-61 for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter that applicant regards as the invention. The Examiner states that a claim may not contain a broad limitation together with a narrow limitation that falls within the range of the broad limitation. Applicants have, with this amendment, brought claims 16, 17, 21, 26, 27, 29, 36, 39 and 52 into proper form by removing the narrow limitation expressed within each. Additionally, new dependent claims 63 - 74 have been added which each recite the corresponding narrow limitation explicitly. The Examiner also rejected Claims 58-61 as being indefinite for apparently the same reason but has not provided reasons in the Office Action mailed October 5th, 1999. Applicants therefore respectfully request clarification of this alleged rejection of Claims 58-61.

Applicants have amended Claim 1 to more particularly recite the linking arrangement between the peptide and the nucleic acid. Applicants have further added claim 82 directed towards a method of use for the chimeric peptide-nucleic acid fragment.

Applicants have amended the language of Claims 3, 4, 8, 17, 19, 25, 28, 29, 34, 44, 45, 46, 51 and 54 to use the terms "comprises", "comprise" or "comprising", instead of "has", "have" or "having". Claims 35 and 36 have been amended to overcome other rejections and no longer use the terms "has" and "having", respectively.

Claim 6 has been amended to describe a nucleic acid portion of the claimed chimera which is capable of hybridizing to form a linear single-strand structure. This embodiment is described in page 14 of the substitute specification filed herewith.

Applicants have amended Claim 7 to utilize a Markush grouping, thereby removing use of the term "preferably".

Applicants have amended Claim 10 to replace the word "when" with "and", so that the chimeras encompassed by the Claim can be determined. Claims 11 and 17 have been similarly amended.

Applicants have amended Claim 12 to describe a linkage group "bound via at least one C2 spacer." Applicants have entered a new dependent Claim 78 which recites the use of a linkage group "bound via a C6 spacer."

Applicants have amended Claim 13 to utilize a Markush grouping of two alternative locations for the linking group. The attachment of the linking group to the base position is claimed in new claim 79.

Applicants have amended Claim 14 to utilize a Markush grouping of two alternative locations for the linking group and a Markush grouping for the several alternative types of amino acid.

Applicants have amended Claim 20 to recite an "additional" cysteine in place of an "artificial" cysteine. At pages 5 and 22 of the substitute specification filed herewith, description can be found of peptide sequences which are extended by an additional cysteine residue at their termini in order to facilitate linkage.

Applicants have amended Claim 22 to remove the alleged indefiniteness. The functional groups recited are now listed in Markush format and it is stipulated that both the linkage group and the signal peptide or nucleic acid contain one of the functional groups.

Applicants have amended Claim 24 in the manner suggested by the Examiner.

Claim 31 has been amended to more particularly claim the transcription control elements associated with transcription of a mitochondrial genome.

Claim 32 has been amended to more particularly recite the elements of L-strand transcription. Support for this form of transcription can be found on page 17 of the substitute specification filed herewith.

Claim 36 has been amended and dependent claim 70 has been added to more particularly claim H-strand transcription control. Support for this form of transcription can be found on page 18 of the substitute specification filed herewith.

Claim 35 has been amended, as described above, to avoid the use of the word "preferably".

Applicants have amended Claim 41 to delete the material, "preferably ... on the plasmid" and presented similar material as a further limitation in new claim 80. Support for

this amendment may be found on page 18 of the specification. Claim 41 has also been amended to depend from Claim 40 so as to correct the lack of antecedent basis for "multiple cloning sites".

Claims 30, 42, 43 have been amended to replace the term 'direction' with the term 'side'. Even in a cyclized nucleic acid, it is possible to distinguish the 3' from the 5' sides of each nucleotide.

Claim 44 has been amended to recite the form of the ligation envisaged.

Claims 45, 46 and new Claims 75 and 76 have been amended to specify that the properties of the nucleic acid ends pertain prior to cyclisation.

Claim 50 has been amended to delete the word "preferably" and also to replace the term 'loop' by the term 'cyclic portion'.

Claim 51 has been amended to delete the material, "preferably ... in the plasmid sequence". The deleted limitation has been presented in new dependent claim 77.

Claim 52 has been amended to delete the material, "preferably ... recognition sequence". The deleted limitations have been presented in new dependent claims 73 and 74.

Claim 56 has been amended to delete the optional step. The optional step has been included in new Claim 81. Claim 57 has been amended to depend from Claim 81, thereby obviating the lack of antecedent basis.

Claims 60 and 61 have been amended to recite a "method of introducing" peptide-nucleic acid fragments into eukaryotic cells.

Claims 58 and 59 have been amended to recite a method of introducing the chimerical peptide-nucleic acid fragment into cells comprising a single step.

Claims 25, 33, 37, 39, 40, 41 and 51 have been rejected because the use of the term 'plasmid' allegedly does not correspond to the usual meaning of the term. Applicants have substituted the term "molecule" for "plasmid". A construct of a nucleic acid, a peptide and a linkage agent when bound together covalently may reasonably be called a "molecule".

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-61 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in sucg a way to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention. The rejection is respectfully traversed.

Specifically, Claims 1, 18, 19 and 25 were rejected because "cell-specific, compartment-specific or membrane-specific peptides" were allegedly not disclosed in the specification. Claims 1, 18, 19 and 25 as amended, have been limited to cell compartments taken from the group consisting of mitochondria and chloroplasts, thus rendering this rejection moot.

Claim 14 has been rejected because the specification allegedly does not disclose genes that would be targeted by the claimed anti-sense oligonucleotides. The reference to anti–sense oligonucleotides has been deleted from Claim 14 as amended, thereby rendering this rejection moot.

Claims 32 and 36 have been rejected because the structure of the "conserved sequence blocks" allegedly cannot be determined. The term "conserved sequence blocks" has been well known in the art for a very long time (see for example, Chang *et al.*, (1985) *Proc. Natl. Acad. Sci. USA*, 82:351-355). Moreover, given a particular sequence, it would be straightforward for one skilled in the art to determine which sequences are the "conserved sequence blocks". For example, in Chang *et al.*, Conserved Sequence Blocks I, II and III are shown in figure 5. Therefore, claims 32 and 36, as presented are sufficiently definite that one of skill in the art could determine what is meant.

Claim 37 has been rejected because it recites a chimera containing "regulatory sequence", a term which the Examiner alleges is not determined from the specification. Claim 37, and also claims 27, 28, 29 and 30 have been amended to recite the term "regulation" in place of "regulatory". The specification describes transcription regulation sequences at page 17.

Claim 38 has been cancelled without prejudice.

Claims 1 and 58-61 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for methods of using one specific type of peptide-nucleic acid chimera for in vitro delivery of a nucleic acid to the mitochondria, does not reasonably provide enablement for methods of use of that peptide-nucleic acid to deliver a nucleic acid to mitochondria in vitro. The rejection is respectfully traversed.

Applicants have disclosed a novel chimera and method of introducing the same into mitochondria. Applicants have further disclosed, in Example 2, a method of introducing the chimera into the mitochondria of living cells. At the time of filing, there were many methods

which would have been known to one skilled in the art of introducing a chimera into cells, *in vivo*. For example, the use of liposomes in direct gene transfer has been reported (Nabel, *et al.*, *Proc. Natl. Acad. Sci. USA*, (1993), 90:11307-11311). In another approach, known as *ex vivo* gene therapy, cells are genetically corrected outside the body and reinserted, (see for example, Grossman *et al.*, *Nature Genetics*, (1994), 6:335-341). In yet another approach, an adenovirus is employed to introduce a fragment of DNA into the body (see for example, Crystal *et al.*, *Nature Genetics*, (1994), 8:42-51). In a further approach, lymphocytes are employed, (see for example, Blaese *et al.*, *Science*, (1995), 270:475-480). Therefore, Applicants respectfully submit that, according to a number of techniques which were widely known and would have been understood by one skilled in the art, it would have been possible to practice the disclosed invention on living organisms.

Claims 1 and 58-61 have been rejected because the Examiner alleges that peptides that require residues at or near the carboxyl terminus will not necessarily be able to utilize cellular transport mechanisms. According to Claim 1, as amended, recites a chimera which is capable of entering a cellular compartment, thereby rendering this rejection moot.

Claim 1 and 58-61 have been rejected for allegedly reading on nucleic acids whose secondary or tertiary structure does not permit them to function compatibly with cellular transport mechanisms. Claim 1, as amended, however, recites a nucleic acid which is capable of entering a cellular pore. The nucleic acid structures which are consistent with this limitation will not suffer from the difficulties raised by the Examiner.

No new matter has been introduced by the any of the foregoing amendments and entry thereof into the instant application is respectfully requested.

Rejections Under 35 U.S.C. § 102(e)

The Examiner has rejected claims 1-24 and 54-61 as allegedly being anticipated by Lin *et al.* Applicants respectfully traverse the rejection. For a reference to anticipate a claim under 35 U.S.C. § 102(e), the reference must teach each and every limitation of the claim. *See, Scripps Clinic & Research Foundation v. Genentech, Inc.* 18 U.S.P.Q.2d 1001, 1010 (Fed. Cir. 1991) ("Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention." (citations omitted)).

Specifically, Lin *et al.* teaches the delivery of a nucleic acid into the interior of cells and nuclei of cells by chemically linking it to an importation competent signal peptide at the carboxy terminus of the signal peptide. Claim 1 of the instant application, as amended, however, recites a signal peptide whose signal sequence is specific to a cell compartment, particularly *mitochondria* and *chloroplasts*. New Claim 82 recites a method of introducing a nucleic acid into a cell compartment, particularly mitochondria and chloroplasts. Lin *et al.* does not teach the importation of a nucleic acid into cell organelles. Merely teaching the importation of a nucleic acid into a cell or its nucleus, as in Lin *et al.*, therefore does not anticipate the claims of the instant application. Therefore the teaching of Lin *et al.* does not meet all of the limitations of Claims 1-24 and 54-61. Applicants respectfully submit that the rejection of Claims 1-24 and 54-61 under 35 U.S.C. § 102(e) is traversed.

Rejections Under 35 U.S.C. § 103

The Examiner has rejected Claims 1-24 and 58-61 under 35 USC § 103 (a) as being allegedly unpatentable over Lin *et al.* in view of Latham *et al.* and Horwich *et al.* When rejecting claims under 35 U.S.C. § 103, the Examiner bears the burden of establishing *a prima facie* case of obviousness. *In re Bell,* 26 USPQ2d 1529 (Fed. Cir. 1993). To establish a *prima facie* case, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or combine the reference teachings in the manner suggested by the Examiner. *See, e.g., In re Grabiak,* 226 USPQ 870 (Fed. Cir. 1985). Second, the skilled artisan, in light of the teachings of the prior art, must have a reasonable expectation that the modification or combination suggested by the Examiner would be successful. *See, e.g., In re Dow,* 5 USPQ2d 1529, 153 1-32 (Fed. Cir. 1988). Finally, the prior art reference, or references when combined, must teach or suggest each and every limitation of the claimed invention. M.P.E.P. § 706.02(j). The teaching or suggestion to make the claimed invention *and* the reasonable expectation of success must *both* be found in the prior art, not in the Applicants' disclosure. *In re Vaeck,* 20 USPQ2d 1438 (Fed. Cir. 1991). If any one of these criteria is not met, *prima facie* obviousness is not established. Applicant respectfully submits that the cited references, individually and in combination, do not teach or suggest each and every limitation of the invention as claimed.

As already stated, Lin *et al.* teach a nucleic acid linked to a peptide for the purpose of introduction into cells and nuclei of cells. Lin *et al.* do not teach the introduction of a nucleic acid into mitochondria or chloroplasts. The deficiencies of Lin *et al.* are not supplied by . Latham *et al.* or Horwich *et al.*

Latham *et al.* teach the delivery of an oligonucleotide into a cell by linking the oligonucleotide to a "transport agent" via a linkage agent which comprises at least one disulfide bond. Latham *et al.* do not teach the delivery of a nucleic acid into cell compartments such as mitochondria but restrict their teaching to the facilitation of transport across an outer cell membrane or the blood-brain barrier. Furthermore, the "transport agent" defined by Latham *et al.* is not the same as the chimeric peptide-nucleic acid of the present invention. Latham *et al.*, p.22-23, define a "transport agent" to be a number of agents including lipophilic entities, polycations, cholesterol, peptides, proteins, saccharides, antibodies, cellulose, etc. The list of agents disclosed by Latham *et al.* does not comprise a "signal peptide."

Horwich *et al.* teaches that the Ornithine Transcarbamylase (OTC) leader peptide directs mitochondrial import. However, Horwich *et al.* does not teach the use of OTC to import a nucleic acid into mitochondria and does not teach the attachment of OTC to a nucleic acid via a linkage group. Moreover, there is nothing in the teachings of Horwich *et al.* to suggest combination of OTC with a nucleic acid or a linkage agent to produce a chimeric peptide-nucleic acid fragment as disclosed in the present invention. Furthermore, there is nothing in the teaching of either Lin *et al.* or Latham *et al.* to suggest the use of OTC for the introduction of nucleic acids into cells or cell organelles. Therefore there is no suggestion or motivation in the references that would lead one of ordinary skill in the art to modify or combine the reference teachings in the manner suggested by the Examiner

Accordingly, since neither Lin *et al.* nor Latham *et al.*, alone or in combination, teach or suggest the delivery to *mitochondria* recited in Claims 1-24 and 58-61, these references do not render Claims 1-24 and 58-61 *prima facie* obvious. Because of the failure of the Examiner in stating a *prima facie* case of obviousness against Claims 1-24 and 58-61 and the strong evidence of non-obviousness established by the art of record, Applicant respectfully requests that the rejection of Claims 1-24 and 58-61 under 35 U.S.C. § 103(a) be withdrawn.

CONCLUSION

The above amendments are made to comply with the formal requirements set forth in 37 C.F.R. §1.125. They do not introduce new matter, and they are fully supported by the specification of the subject Application and the claims as originally filed. Applicants respectfully request that the above-made amendments be made of record in the file history of the instant application.

The Commissioner is hereby authorized to charge any fees associated with filing this Amendment to Pennie & Edmonds Deposit Account No. 16-1150; the fees are estimated to be $246.00 for adding twenty two (22) new claims to the present application. A copy of this sheet is enclosed.

Respectfully submitted,

Date: April 5, 2000

Laura A. Coruzzi 30,742
Laura A. Coruzzi (Reg No.)
PENNIE & EDMONDS LLP
3300 Hillview Avenue
Palo Alto, California 94304
(650) 493-4935

Exhibit A
Pending Claims
After Entry of Amendment

1. A chimeric peptide-nucleic acid fragment capable of entering a cellular compartment, comprising:

(a) a compartment-specific signal peptide;

(b) a linkage agent; and

(c) a nucleic acid which is capable of entering a cellular pore;

wherein the linkage agent links an amino acid at the carboxy-terminal end of the signal peptide to the nucleic acid and wherein the signal peptide is specific to a compartment selected from the group consisting of mitochondria and chloroplasts.

2. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the nucleic acid comprises at least two bases.

3. The chimeric peptide-nucleic acid fragment according to claim 2, wherein the nucleic acid comprises a secondary structure.

4. The chimeric peptide-nucleic acid fragment according to claim 2, wherein the sequence of the nucleic acid is partially palindromic .

5. The chimeric peptide-nucleic acid fragment according to claim 4, wherein the nucleic acid may form a hairpin loop.

6. The chimeric peptide-nucleic acid fragment according to claim 5, wherein the nucleic acid hybridizes to itself and form a linear single-strand structure with an overhanging 3' end.

7. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the nucleic acid is selected from the group consisting of ribonucleic acid and deoxyribonucleic acid.

8. The chimeric peptide-nucleic acid fragment according to claim 7, wherein the phosphorous diester bonds of the nucleic acid are substituted with phosphorus thioate bonds.

9. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the nucleic acid carries a reactive linkage group.

10. The chimeric peptide-nucleic acid fragment according to claim 9, wherein the reactive linkage group contains an amino function and the linkage agent contains an amino-reactive group.

11. The chimeric peptide-nucleic acid fragment according to claim 9, wherein the reactive linkage group contains a thiol function and the linkage agent contains a thiol-reactive group.

12. The chimeric peptide-nucleic acid fragment according to claim 10 or 11, wherein the linkage group present is bound to the nucleic acid via at least one C2 spacer.

13. The chimeric peptide-nucleic acid fragment according to claim 12, wherein the linkage group is localized at a position selected from the group consisting of the 3' hydroxy/phosphate terminus of the nucleic acid and the 5' hydroxy/phosphate terminus of the nucleic acid.

14. The chimeric peptide-nucleic acid fragment according to claim 12, wherein the nucleic acid is selected from the group consisting of messenger RNAs, transcribable genes and replicatable genes and wherein said nucleic acid is linked at a position selected from the group consisting of the 5' end and the 3' end.

15. The chimeric peptide-nucleic acid fragment according to claim 14, wherein the nucleic acid to be linked comprises phosphorus thioate bonds.

16. The chimeric peptide-nucleic acid fragment according to claim 14, wherein the gene to be linked contains a promoter.

17. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the signal peptide comprises a reactive amino acid at the carboxy-terminal end, and the linkage agent contains an amino-reactive or thiol-reactive group.

18. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the signal peptide carries a compartment-specific recognition sequence specific to a compartment selected from the group consisting of mitochondria and chloroplasts.

19. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the signal peptide comprises a peptidase cleavage site specific to a compartment selected from the group consisting of mitochondria and chloroplasts.

20. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the peptide comprises the compartment-specific cleavable signal peptide of the human mitochondrial ornithine transcarbamylase, extended by an additional cysteine at the C terminus.

21. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the linkage agent is a bifunctional cross-linker.

22. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the linkage agent and at least one of the signal peptide and the nucleic acid each carry a functional group selected from the group consisting of thiol-reactive and amino-reactive groups as linkage sites.

23. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the linkage agent is m-maleimido-benzoyl-N-hydroxy-succinimide ester or a derivative thereof.

24. The chimeric peptide-nucleic acid fragment according to claim 1, wherein the molecule overcomes membranes with and without membrane potential by utilizing natural transport mechanisms.

25. A chimeric peptide-nucleic acid fragment in the form of a linear-cyclic molecule, wherein the molecule comprises at least one replication origin and both ends of the nucleic acid portion are cyclized, and wherein at least one cyclic end comprises a modified nucleotide which via a linkage agent can be linked with a signal peptide specific to a compartment selected from the group consisting of mitochondria and chloroplasts.

26. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the nucleic acid portion further comprises at least one promoter.

27. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the nucleic acid portion further comprises transcription-regulation sequences.

28. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the transcription-regulation sequences comprise at least one binding site of a transcription initiation factor.

29. The chimeric peptide-nucleic acid fragment according to Claim 25, wherein the transcription-regulation sequences comprise at least one binding site for RNA synthesis apparatus.

30. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the transcription-regulation sequences are arranged on the 3' side of the promoter.

31. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the plasmid comprises transcription control elements which are suitable for H-strand and L-strand transcription of a mitochondrial genome.

32. The chimeric peptide-nucleic acid fragment according to claim 31, wherein the transcription control elements suitable for L-strand transcription are conserved-sequence-blocks.

33. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the molecule further comprises at least one transcription termination site.

34. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the transcription termination site comprises a binding sequence of a mitochondrial transcription termination factor.

35. The chimeric peptide-nucleic acid fragment according to claim 34, wherein the binding sequence of the mitochondrial transcription termination factor is bidirectionally acting.

36. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the replication origin is a mitochondrial replication origin.

37. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the molecule further comprises at least one regulation sequence for replication.

39. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the molecule further comprises a selection gene.

40. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the molecule further contains a multiple cloning site which permits the expression of foreign genes.

41. The chimeric peptide-nucleic acid fragment according to claim 40, wherein the multiple cloning site comprises recognition sequences for restriction endonucleases.

42. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the multiple cloning site is arranged in the 3' direction of the promoter and on the 5' side of the transcription termination site.

43. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the multiple cloning site is arranged on the 5' side of the selection gene.

44. The chimeric peptide-nucleic acid fragment according to claim 25 wherein the ends of the nucleic acid fragment are joined to the peptide fragment by ligation.

45. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the nucleic acid fragment prior to cyclisation comprises a blunt end.

46. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the nucleic acid fragment prior to cyclisation comprises a 5' overhang which comprises 4 nucleotides with the proviso that the 4 nucleotides do not have a self-homology and are not complementary to one another.

47. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the ends of the nucleic acid fragment are cyclized via synthetic oligonucleotides.

48. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the overhanging 5' ends of the two oligonucleotides are complementary to one differing end of the nucleic acid each.

49. The chimeric peptide-nucleic acid fragment according to claim 25, wherein two differing hairpin loops are used for the cyclization, one being specific (complementary) to the plasmid end and the other being specific to the plasmid end of the nucleic acid.

50. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the modified nucleotide is localized within the cyclic portion.

51. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the molecule DNA is amplified enzymatically by suitable oligonucleotides which comprise at least one recognition sequence for a restriction endonuclease.

52. The chimeric peptide-nucleic acid fragment according to claim 51, wherein the restriction endonuclease to be used comprises an overhanging end.

53. The chimeric peptide-nucleic acid fragment according to claim 51, wherein the restriction endonuclease is B*sa*I.

54. A process for the production of a chimeric peptide-nucleic acid fragment according to claim 1 or 25, comprising the following steps:

(a) reaction of a nucleic acid (oligonucleotide) containing a functional linkage group which comprises a linkage agent;

(b) reaction of the construct of step (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence; and

(c) optional extension of the chimeric peptide-nucleic acid fragment of (b) by a further fragment selected from the group consisting of DNA or RNA.

55. The process according to claim 54, wherein the DNA in step (c) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR}$).

56. A process for the production of a chimeric peptide-nucleic acid fragment according to claim 1 or 25, comprising the following steps:

(a) reaction of a nucleic acid with a functional linkage group with a linkage agent,

(b) reaction of the construct of (a) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence.

57. The process according to claim 81, wherein the DNA fragment in step (i) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR}$).

58. A method of using the chimeric peptide-nucleic acid fragment [according to] of claim 1 or 25 for introducing the nucleic acid into cells and mitochondria, comprising the step of reacting the fragment with cells or pretreated mitochondria.

59. The method of claim 58, wherein the pretreated cell compartments are energized mitochondria.

60. A method of introducing the chimeric peptide-nucleic acid fragment according to claims 1 or 25 into eukaryotic cells.

61. The method according to claim 60, comprising the particle gun system, electroporation, microinjection or lipotransfection for introducing the chimeric peptide-nucleic acid fragment into eukaryotic cells.

62. The chimeric peptide-nucleic acid fragment according to claim 44 wherein the ends of the nucleic acid fragment are phosphorylated.

63. The chimeric peptide-nucleic acid fragment according to claim 16, wherein the promoter is a mitochondrial promoter.

64. The chimeric peptide-nucleic acid fragment according to claim 17, wherein the reactive amino acid at the carboxy-terminal end is a lysine or cysteine.

65. The chimeric peptide-nucleic acid fragment according to claim 21, wherein the linkage agent is a heterobifunctional cross-linker.

66. The chimeric peptide-nucleic acid fragment according to claim 26, wherein the promoter is a mitochondrial promoter.

67. The chimeric peptide-nucleic acid fragment according to claim 26, wherein the promoter is a mitochondrial promoter of the light strand.

68. The chimeric peptide-nucleic acid fragment according to claim 27, wherein the transcription-regulatory sequences are mitochondrial transcription-regulatory sequences.

69. The chimeric peptide-nucleic acid fragment according to Claim 29, wherein the binding site for the RNA synthesis apparatus comprises a binding site for the mitochondrial transcription factor 1 and a binding site for the mitochondrial RNA polymerase.

70. The chimeric peptide-nucleic acid fragment according to claim 36, wherein the mitochondrial replication origin is the replication origin of the heavy mtDNA strand and comprises at least one conserved sequence block.

71. The chimeric peptide-nucleic acid fragment according to claim 39, wherein the selection gene is an antibiotic-resistance gene.

72. The chimeric peptide-nucleic acid fragment according to claim 39, wherein the selection gene is the oligomycin-resistance or chloramphenicol-resistance gene.

73. The chimeric peptide-nucleic acid fragment according to claim 52, wherein the restriction endonuclease to be used comprises a 5' overhanging end.

74. The chimeric peptide-nucleic acid fragment according to claim 52, wherein the restriction endonuclease to be used comprises a cleavage site localized preferably outside the recognition sequence.

75. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the nucleic acid fragment prior to cyclisation comprises an overhanging 3' end.

76. The chimeric peptide-nucleic acid fragment according to claim 25, wherein the nucleic acid fragment prior to cyclisation comprises an overhanging 5' end.

77. The chimeric peptide-nucleic acid fragment according to claim 51, wherein the recognition sequence for a restriction endonuclease occurs in non-repeated fashion in the molecule sequence.

78. The chimeric peptide-nucleic acid fragment according to claim 10 or 11, wherein the linkage group present is bound to the nucleic acid via a C6 spacer.

79. The chimeric peptide-nucleic acid fragment according to claim 12, wherein the linkage group is localized at the position of a modified nucleoside base on the nucleic acid.

80. The chimeric peptide-nucleic acid fragment according to claim 40, wherein the recognition sequences for restriction endonucleases of which the multiple cloning site is comprised do not occur in another site of the molecule.

81. A process for producing a chimeric peptide-nucleic acid fragment according to claim 1 or 25, comprising the steps:

(i) extension of a nucleic acid containing a functional linkage group by a fragment selected from the group consisting of DNA and RNA;

(ii) reaction of the fragment of step (i) with a linkage agent; and

(iii) reaction of the construct of (ii) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence.

82. A method of introducing a nucleic acid with a functional linkage group into a compartment of a cell, comprising the steps:

(a) reaction of the nucleic acid with a linkage agent;

(b) reaction of the construct of step (a) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence, to form a chimeric peptide-nucleic acid fragment; and

(c) contacting the chimeric peptide-nucleic acid fragment of step (b) with the cell;

wherein the signal sequence of the peptide is specific to a cell compartment selected from the group consisting of mitochondria and chloroplasts.

83. The chimeric peptide-nucleic acid fragment according to claim 5, wherein the nucleic acid hybridizes to form a linear single-strand structure with an overhanging 5' end.

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Application of: P. Seibel and A. Seibel

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For: CHIMERICAL PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USES FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

STATEMENT ACCOMPANYING SUBSTITUTE SPECIFICATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Pursuant to 37 C.F.R. § 1.125, submitted herewith is a Substitute Specification. The Substitute Specification differs from the English translation of the original German Specification in that it has been reformatted to comply with U.S. practice. A marked-up copy of the Substitute Specification is provided to illustrate the differences from the English translation of the original German Specification. The Substitute Specification does not contain new matter. Entry of the substitute specification is kindly solicited.

No fee is believed due. However, if it is determined that additional fees are due, please charge them to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed for accounting purposes.

Respectfully submitted,

Date April 5, 2000

Laura A. Coruzzi 30,742
Laura A. Coruzzi (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

Enclosures

Sent Appc

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

CHIMERIC PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USE FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

This is a national phase filing of the Application No. PCT/DE95/00775, which was filed with the Patent Corporation Treaty on June 11, 1995, and is entitled to priority of the German Patent Application P 44 21 079.5, filed June 16, 1994.

I. FIELD OF THE INVENTION

This invention relates to a chimeric peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

II. BACKGROUND OF THE INVENTION

It is known that cellular membrane systems are largely impermeable to nucleic acids. However, cell membranes can be penetrated very efficiently by physical processes (transformation) and biological processes (infection).

Transformation, i.e., the direction absorption of naked nucleic acid by cells, is preceded by cell treatment. There are various methods available for the production of these "competent cells". Most processes are based on the observations made by Mandel and Higa (M. Mandel *et al.*, (1970), "Calcium-dependent bacteriophage DNA infection", *J. Mol. Biol.* 53: 159-162), who managed to show for the first time that yields resulting from the absorption of λ-DNA by bacteria can be increased significantly in the presence of calcium chloride. This method was also used successfully for the first time by Cohen *et al.* (S.N. Cohen *et al.* (1972), "Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA", *Proc. Natl. Acad. Sci. U.S.A.* 69:2110-2114) for plasmid DNA and was improved by many modifications (M. Dagert *et al.* (1979), "Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells", *Gene* 6:23-28). Another transformation method is based on the

observation that high-frequency alternating fields may break up cell membranes (electroporation). This technique can not only be used to introduce naked DNA into prokaryotic cells but also eukaryotic cell systems (K. Shigekawa *et al.* (1988), "Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells", *Biotechniques* 6:742-751). Two very gentle methods of introducing DNA into eukaryotic cells were developed by Capecchi (M.R. Capecchi (1980)), "High efficiency transformation by direct microinjection of DNA into cultured mammalian cells" *Cell* 22:479-488) and Klein *et al.* (T.M. Klein *et al.* (1987), "High velocity microprojectiles for delivering nucleic acids into living cells", *Nature* 327:70-73). They are based, respectively, on the direct injection of the DNA into the individual cell (microinjection), and on the bombardment of a cell population with microprojectiles consisting of tungsten, to the surface of which the corresponding nucleic acid is bound ('shotgun').

The biological infection methods have proved their value concurrently with the physical transformation of cells. In particular, they include the highly efficient viral introduction of nucleic acids into cells (K.L. Berkner (1988), "Development of adenovirus vectors for the expression of heterologous genes", *Biotechniques* 6:616-629; L.K. Miller (1989), "Insect baculoviruses: powerful gene expression vectors", *Bioessays* 11:91-95; B. Moss *et al.* (199), "Product review. New mammalian expression vectors", *Nature* 348:91-92) and the liposome mediated lipofection (R.J. Mannino *et al.* (1988), "Liposome mediated gene transfer", *Biotechniques* 6:682-690; P.L. Felgner *et al.* (1987), "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure", *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417).

All methods described so far deal with overcoming the prokaryotic or eukaryotic plasma membrane by naked or packaged nucleic acids. While the site of action is reached immediately when the nucleic acids are introduced into a prokaryotic cell, further biochemical processes take place in a compartmentalized eukaryotic cell, which allow the penetration of the nucleic acid into the nucleus under certain conditions (e.g., viral route of infection in the case of HIV). Analogous infective processes in which exogenous nucleic

acids are actively introduced into other cell organelles (e.g., into mitochondria) have not been described so far.

In addition to the introduction of the nucleic acid into the cell and cell organelle, respectively, the transcription and particularly the replication of the introduced nucleic acid play a decisive role. In this connection, it is known that DNA molecules may have a special property which permits duplication in a cell under certain conditions. A special structural element, the origin of the DNA replication (also known as "ori", or "origin"), adds thereto. Its presence provides the ability of DNA replication (K.J. Marians (1992), "Prokaryotic DNA replication", *Annu. Rev. Biochem.* 61:673-719; M.L. DePamphilis (1993), "Eurkaryotic DNA replication: anatomy of an origin", *Annu. Rev. Biochem.* 62:29-63; H. Echols and M.F. Goodman (1991), "Fidelity mechanisms in DNA replication", *Annu. Rev. Biochem.* 60:477-511). The strictly controlled process of DNA replication starts in E. coli e.g., when a protein is bound (K. Geider and H. Hoffman Berling (1981), "Proteins controlling the helical structure of DNA", *Annu. Rev. Biochem.* 50:233-260) to the highly specific initiation site thus defining the starting point of a specific RNA polymerase (primase). It synthesizes a short RNA strand (~10 nucleotides, 'primer') which is complementary to one of the DNA template strands. The 3' hydroxyl group of the terminal ribonucleotide of this RNA chain serves as a "primer" for the synthesis of new DNA by a DNA polymerase. DNA-untwisting proteins unwind the DNA double helix (J.C. Wang (1985), "DNA topoisomerases", Annu. Rev. Biochem. 54:665-697). The separated individual strands are stabilized by DNA-binding proteins as regards their conformation (J.W. Chase and K.R. Williams (1986), "Single-stranded DNA binding proteins required for DNA replication", *Annu. Rev. Biochem.* 55:103-136) to enable proper functioning of the DNA polymerases (T.S. Wang (1991), "Eukaryotic DNA polymerases", *Annu. Rev. Biochem.* 60:513-552). A multienzyme complex, the holoenzyme of DNA-polymerase-III, synthesizes the majority of the new DNA. The RNA portion of the chimeric RNA-DNA molecule is then split off the DNA polymerase III. The removal of the RNA from the newly formed DNA chains creates gaps between the DNA fragments These gaps are filled by the DNA-polymerase I which can newly synthesize DNA from a single-stranded template. While one of the two newly synthesized DNA strands is synthesized

continuously (5'-3' direction, leader strand), Ogawa and Okazaki observed that a majority of the newly synthesized opposite strand (3'-5' direction, delayed strand) was synthesized from short DNA fragments (T. Ogawa and T. Okazaki (1980), "Discontinuous DNA replication", *Annu. Rev. Biochem.* 49:421-457). Here, what are called primases initiate the onset of the DNA synthesis of the opposite strand by the synthesis of several RNA primers. When the replication proceeds, these fragments are freed from their RNA primers, the gaps are closed and covalently linked with one another to give extended daughter strands by the DNA ligase. Two chromosomes form after the termination of the replication cycle.

As opposed thereto, the DNA replication is controlled by many plasmids via a replication origin which dispenses with the synthesis of the delayed strand (3'-5' direction) and can initiate the synthesis of two continuous DNA strands bidirectionally (each in the 5'-3' direction along the two templates). The precondition for complete DNA replication here is the cyclic form of the nucleic acid. It ensures that at the end of the new synthesis of the complementary DNA strands the DNA polymerases return to the starting point again where ligases now guarantee the covalent linkage of the ends of the two newly synthesized daughter strands.

Smallpox viruses represent an interesting form of linear-cyclic nucleic acids: because of what is called "hairpin loops" at the ends of their linear genomes their molecules have a cyclic structure while maintaining a predominantly linear conformation (D.N. Black *et al.* (1986), "Genomic relationship between capripoxviruses", *Virus Res.* 5:277-292; J.J. Esposito and J.C. Knight (1985) "Orthopoxyvirus DNA: a comparison of restriction profiles and maps", *Virology* 143:230-251). Covalently closed "hairpin" nucleic acids were not only found in smallpox viruses but also described for the ribosomal RNA from Tetrahymena (E.H. Blackburn and J.T. Gall (1978), "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena", *J. Mol. Biol.* 120:33-53) and the genomes of the parvoviruses (S.E. Straus *et al.* (1976), "Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis", *Proc. Natl. Acad. Sci. U.S.A.* 73:742-746; P. Tattersall and D.C. Ward (1976), "Rolling hairpin model for the replication of parvovirus and linear chromosomal DNA", *Nature* 263:106-109).

However, it is not possible to introduce nucleic acids into cells or cell organelles adequately using the protein import route by means of the formerly known plasmids or nucleic acid constructs. But such an approach is, for example, a precondition for treating genetic changes for the mitochondrial genomes of patients suffering from neuromuscular and neurodegenerative diseases or for carrying out an appropriate mutagenesis in mitochondria or other cell organelles.

III. SUMMARY OF THE INVENTION

This invention relates to a chimeric peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is explained particularly the figures, wherein:

Figure 1 depicts a signal peptide of the ornithine transcarbamylase of rats as well as a DNA sequence suitable for introduction into a cell organelle. Top: signal peptide of the ornithine transcarbamylase of rats (32 amino acids), extended by 10 N-terminal amino acids of the matured protein and an additional cysteine as linkage site (SEQ ID NO:1). The peptide sequence is shown in the international one-letter code; middle: a partially palindromic DNA sequence suitable for introduction and consisting of 39 nucleotides having an amino-modified T at nucleotide position 22 (SEQ ID NO:2); bottom: marked secondary structure of the oligonucleotide having an overhanging 5' end and a modified nucleotide in the vertex of the 'loop'.

Figure 2 depicts the structure of the amino-modified 2-deoxythymidine, R: nucleic acid residues.

Figure 3 depicts a diagram of chimeric peptide-nucleic acid fragment, consisting of amino-modified oligonucleotide (39 nucleotides) with marked 'hairpin loop', cross-linker and signal peptide. CL: Cross=linker.

Figure 4 the electrophoretic separation of the linkage product resulting from amino-modified oligonucleotide (39 nucleotides), m-maleimidobenzoyl-N-hydroxy-succinimide

ester (MBS) and signal peptide of the ornithine transcarbamylase of rats (42 amino acids, extended by a cysteine at the C terminus).

Figure 5A depicts a flow diagram of the peptide-DNA fusion, cloning, amplification and linkage of the transcribable and processable mitochondrial tRNA gene to be introduced (S. Anderson et al. (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465) (SEQ ID NOS:2-6). CL: cross-linker (MBS); MCS: multiple cloning site of pBluescript^{R} (Stratagene), mtTF: binding site of the mitochondrial transcription factor; RNA-Pol: binding site of the mitochondrial RNA polymerase; tRNA Leucin: gene of the mitochondrial transfer RNA for leucine (UUR); *Sac II, Apa I, Eco RI*: sites for restriction endonucleases; the cloned mitochondrial sequences were numbered in accordance with the published sequence of the human mitochondrial genome (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465)

Figure 5B and 5C depicts the sequence of the cloned $tRNA^{Leu(UUR)}$ gene (SEQ ID NOS:7 and 8).

Figure 6A and 6B depict a representation of the ^{32}P radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitochondria-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 7A and 7B depicts a representation of the ^{32}P radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitoplast-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient, is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 8 depicts the cloning of the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 1). Using the two oligonucleotides (primers 1 and 2), the gene section of nucleotide 15903 to nucleotide 677 was amplified enzymatically from

mitochondrial HeLa DNA (comprises: promoter characterized by the binding sites for the mitochondrial transcription factors and the RNA polymerase; replication origin characterized by what is called 'conserved sequence blocks'; regulation of the DNA replication characterized by the 'TAS' motifs). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Xho* I and *Pst* I, the ends of the amplified nucleic acid can be modified such that they are compatible with a vector arm of pBluescript, on the one hand, and compatible with the hybrid of the oligonucleotides MCS/TTS 1 and 2, on the other hand. In addition to a multiple cloning site, they also comprise a transcription termination sequence which is responsible for the regulated transcription. The ligation product is then transformed into *E. coli* XL 1. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to RFLP and sequence analysis.

Figure 9 depicts the sequence of the oligonucleotides MCS/TTS 1 and 2 (SEQ ID NOS:9 and 10). The oligonucleotides MCS 1 and 2 were prepared synthetically and comprise recognition sequences for nine different restriction endonucleases as well as a sequence motif which can suppress the transcription bidirectionally. The olignucleotides are complementary and can thus form a hybrid. The overhanging ends are part of the recognition sequences for the restriction endonucleases *Pst*I and *Bam* HI.

Figure 10 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 1) (SEQ ID NOS:11 and 12).

Figure 11 depicts the cloning of the reporter gene into the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 2). Using the two oligonucleotides (primers 3 and 4), the gene section of nucleotide 1562 to nucleotide 3359 was amplified enzymatically from a DNA extract of a human CAP=resistant cell line (comprises: part of the 12 S rRNA gene, $tRNV^{Val}$ gene, 16 S $rRNA^{CAP+}$ gene, $tRNA^{Leu}$ gene, part of ND 1 gene). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Hind* III and *Bcl* I, the ends of the amplified nucleic acid can be modified such that they are compatible with the multiple cloning site (MCS) of the peptide-nucleic acid plasmid (plasmid 1). The ligation product is then transformed in *E. coli* XL 1 Blue. Following the

plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to the RFLP and sequence analysis and are available for the described experiments.

Figure 12 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid including the reporter gene (plasmid 2) (SEQ ID NOS:13 and 14).

Figure 13A depicts the reaction run of the cyclization of the nucleic acid portion as well as the conjugation of the nucleic acid portion with a signal peptide. The nucleic acid portion of the peptide-nucleic acid plasmid can be obtained via a plasmid preparation or an enzymatic amplification. In both cases, the treatment with the restriction endonuclease *Bsa* I results in an intermediate product capable of ligation. It can be reacted directly with the monomerized 'hairpin loops'. The reaction product is freed by an exonuclease III treatment from non-specific (non-cyclic) reaction products and educts, is purified and conjugated with the signal peptide via a cross-linker. As an alternative, one of the two 'hairpin loops' can first be conjugated with the signal peptide via a cross-linker before the cyclizing ligation reaction is carried out. A purification of the reaction product follows an exonuclease III treatment here as well.

Figure 13B depicts the structure and sequence of the 'hairpin loop' oligonucleotides HP 1 (SEQ ID NO:2) and 2 (SEQ ID NO:15).

V Figure 14 depicts the monomerization of a 'hairpin loop' oligonucleotide. The synthetic 'hairpin loops' HP 1 and 2 can be monomerized by a thermal or alkaline denaturation. This figure shows a standard agarose gel: lane 1, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III), lane 2: HP 1, synthesis product; lane 3: HP 1, thermally monomerized.

Figure 15 depicts a ligation reaction between the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 2) and the 'hairpin loops' HP 1 and 2. This figure shows a standard agarose gel: lane 1, cloned nucleic acid portion of the peptide-nucleic acid portion in pBluescript treated with the restriction endonuclease *Bsa* I, lane 2: ligation of the reaction products resulting from lane 1 with the 'hairpin loops' HP 1 and 2; lane 3, treatment of the reaction production resulting from lane 2 with exonuclease III; lane 4, molecular weight standard (λDNA treated with the restriction endonucleases *HIND* III and *Eco* RI).

Figure 15B depicts the examination of the purified ligation product by a *Mae* III-RFLP analysis. This figure illustrates a standard agarose gel: lane 1, enzymatically amplified nucleic acid portion following a *Mae III* treatment; lane 2: purified ligation product of the enzymatically amplified nucleic acid portion following a *Mae* III treatment; lane 3: purified product of the plasmid DNA ligation following a *Mae* III treatment; lane 4, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III).

Figure 16 depicts the transcription and replication of the peptide-nucleic acid plasmid. This figure illustrates a standard agarose gel: lane 1, molecular weight standard (λDNA treated with the restriction endonucleases *Hind* III and *Eco* RI); lane 2, untreated peptide-nucleic acid plasmid; lane 3: *in vitro*-obtained transcription products of the peptide-nucleic acid plasmid; lane 4: *in vitro*-obtained replication and transcription products of the peptide-nucleic acid plasmid; lane 5, *in vivo*-obtained replication and transcription products of the peptide nucleic acid plasmid; lane 6, untreated peptide-nucleic acid plasmid.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to develop a construct on a nucleic acid basis which permits the appropriate introduction of nucleic acids into cell and compartments of eukaryotic cells. Furthermore, a process is to be provided of how this construct can reach cell compartments or cells. In addition, the introduced nucleic acid should be such that it can also be incorporated as replicative nucleic acid via cellular protein import routes. Additionally, properties should be present which result in a controlled transcription and/or replication in cells and in defined targeted compartments of cell, respectively. The process is to be used for the therapy of genetic diseases (changes of the mitochondrial genome) and for the appropriate mutagenesis in eukaryotic and prokaryotic cells. The invention is intended to meet the following demands:

- universal applicability;
- cell-specific, compartment-specific and membrane-specific introduction capability;
- high degree of effectiveness;

- low immunogenicity;
- minimization of the infection risk;
- the introduced nucleic acid (plasmid molecule) is to be replicatable;
- the introduced nucleic acid (plasmid molecule) is to be transcribable;
- the introduced nucleic acid (plasmid molecule) shall be resistant to nucleases; and
- the structure of the introduced nucleic acid (plasmid molecule) should be universally usable.

This problem is solved by the features of claims 1), 25), 54), 56), 58), 60) and 61). Advantageous embodiments follow from the subclaims.

In order to be able to appropriately carry a protein within a cell from the site of formation to another compartment or another cell organelle (e.g. the site of action), the protein is usually synthesized as a preprotein (R. Zimmermann et al. (1983), "Biosynthesis and assembly of nuclear-coded mitochondrial membrane proteins in Neurospora crassa", *Methods Enzymol.* 97:275-286). In addition to the matured amino acid sequence, the preprotein has what is called a signal sequence. This signal sequence is specific to the targeted compartment and enables the preprotein to be recognized by surface receptors. The natural obstacle that the "membrane" presents is then overcome by translocating the preprotein through the membrane by an active process (in which several 'transport proteins' are involved) or a passive process (i.e., direct passage without involvement of further proteins). Thereafter, the signal sequence is usually separated on the site of action by a specific peptidase unless it is a constituent of the matured protein. The matured protein can now unfold its enzymatic activity.

The inventors have recognized that this mechanism can appropriately be utilized to transport nucleic acids across membranes. In this case, the nucleic acid is not subject to a restriction, i.e., it is possible to use every nucleic acid desired and known, respectively. For this purpose, a cell-specific, compartment-specific or membrane-specific signal sequence is linked with the desired nucleic acid, resulting in a chimeric peptide-nucleic acid fragment. In this context, it is known that the linkage between a nucleic acid and a peptide may occur via the α-amino group of a synthetic KDEL (SEQ ID NO:16) peptide, modified by ϵ-

maleimidocapronic acid-N-hydroxysuccinimide ester (K. Arar *et al.* (1993), "Synthesis of oligonucleotide-peptide conjugates containing a KDEL signal sequence", *Tetrahedron Lett.* 34:8087-8090). However, this linkage strategy is completely unusable for the introduction of nucleic acids into cell organelles and cells, since here the translocation should occur in analogy to the natural transport of proteins. Such a transport cannot be expected by blocking the α-amino group of a synthetic peptide by means of a nucleic acid. Therefore, the inventors chose linkage via a carboxy-terminal amino acid. On the one hand, this ensures a 'linear' linkage, on the other hand, the free amino-terminal end of the signal peptide is thus available for the essential steps of the import reaction.

In order to be able to utilize the described transport mechanism also for the introduction of replicative and transcription-active nucleic acids, the nucleic acid is preferably integrated via a homologous recombination into an existing genome or is itself the carrier of the genetic elements, which ensures an autonomous initiation of replication and transcription. Only the latter variant complies with the criterion of universal applicability, since a recombination into an existing cellular genome is successful only under certain conditions and in select cells.

In this case, the use of cyclic DNA represents one possibility, since the DNA polymerases at the end of the new synthesis of the daughter strands return to the initial point thus guaranteeing a complete DNA replication. Although the use of a double-stranded cyclic plasmid meets all physical criteria for a successful replication in every targeted compartment of the cell, this physical DNA form is confronted with the import pore size which is decisively involved in the appropriate translocation: Even the compact diameter of a superhelical plasmid can be compared with that of globular proteins, therefore, a translocation through a membrane system via the protein import route appears impossible. Here, an approach to a solution involves the use of linear-cyclic DNA molecules having modified (cyclic) ends but whose diameter is only the size of linear DNA molecules. On the one hand, they are not too large to go through the import pore; on the other hand, these linear-cyclic DNA molecules include all physical preconditions to be able to form replicative and transcription-active plasmids in the mitochondria.

The following is preferably required for the construction of the chimeric peptide-nucleic acid fragment according to the invention as well as for the construction of a replicative and transcription-active nucleic acid portion (plasmid):

- signal peptide and signal sequence, respectively, (cell-specific, compartment-specific, or membrane-specific);
- linkage agent; and
- nucleic acid (oligonucleotide) which may preferably comprise the following additional information:
  - information in the initiation and regulation of transcription and replication,
  - information as to the termination of transcription and replication,
  - multiple cloning site for a nucleic acid to be introduced (to be expressed) additionally,
  - possible modifications, so that 'hairpin loops' can be added (cyclization of the ends) which permit linkage with the signal peptide.

The selection of the signal sequence depends on the membrane and membrane system, respectively, which is to be overcome and the targeted compartment of the cell (cell nucleus, mitochondrion, chloroplast) or the cell organelle which is to be obtained. Proteins which are to be introduced, e.g., into one of the four mitochondrial compartments (outer mitochondrial membrane, intermembraneous space, inner mitochondrial membrane, matrix space), have compartment-specific signal sequences. In general, signal sequences are chosen for the introduction of nucleic acids which contain a cell specific, compartment-specific or membrane-specific recognition signal thus directing the attached nucleic acid of its site of action (e.g., inner side of the inner mitochondrial membrane or matrix space). A selection can be made among signal sequences which can transport proteins in the presence or absence of a membrane potential.

For the introduction of nucleic acid, signal sequences which function irrespective of the membrane potential are preferred, e.g., the signal sequence of ornithine transcarbamylase (OTC) for the transport into the matrix space of the mitochondria (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rate ornithine transcarbamylase", *Proc. Natl. Acad. Sci. U.S.A.* 80:4258-4262; J. P. Kraus *et al.* (1985), "A cDNA clone for the precursor of rate mitochondrial ornithine transcarbamylase: comparison of rate and human leader sequences and conservation of catalytic sites", *Nucleic. Acids. Res.* 13:943-952). Basically, the pure signal sequence suffices for the transport into the targeted compartment. However, preferable is to select signal sequences which additionally have a cell-specific or compartment-specific peptidase cleavage site. In the most favorable case, the "cleavage site" is within the signal sequence but can also be attached thereto by additional amino acids to ensure the cleavage of the signal sequence when the targeted compartment has been reached (e.g., the signal sequence of human OTC can be prolonged by ten additional amino acids of the matured OTC). This ensures that the nucleic acid can be separated from the signal peptide in the targeted compartment, so that the action of the nucleic acid fully unfolds. The selected signal sequence is prepared biologically (purification of natural signal sequences or cloning and expression of the signal sequence in a eukaryotic or prokaryotic expression system) but preferably in a chemical-synthetic way.

In order to ensure a linear chemical linkage between nucleic acid and signal peptide, the signal peptide is linked via a linkage agent which is generally linked therewith via amino acids, preferably via amino acids having reactive side groups, preferably via an individual cysteine or lysine at the carboxy-terminal end of the signal peptide. A bifunctional cross-linker serves as a linkage reagent, preferably a heterobifunctional cross-linker which has a second reactive group, preferably an aminoreactive group, in addition to a thiol-reactive group at the signal peptide when a cysteine is used as the linkage site (e.g. m-maleinimidobenzoyl-N-hydroxy-succinimide ester, MBS and its derivatives).

The nucleic acid also has a linkage site which should be compatible with the selected cross-linker. When MBS is used, the oligonucleotide should have an amino function or thiol function. The linkage group of the nucleic acid can be introduced via the

chemical synthesis of the oligonucleotide and is generally localized at the 5' end, at the 3' end, but preferably directly at a modified base, e.g., as 5' amino linker (TFA amino linker Amidite^R, 1,6-(N-trifluoroacetylamino)-hexyl-β-cyanoethyl-N,N-diisopropyl phosphoramidite, Pharmacia) or a 5' thiol linker (THIOL-C6 Phosphoramidit^R, MWG Biotech) at a free 5' hydroxy/phosphate group, as 3' amino linker (3' aminomodifier-C7-CPG-Synthesesäulen^R, MWG Biotech) at a free 3' hydroxy/phosphate group, but preferably as amino-modified base analog, preferably amino-modified deoxyuridine (Amino-Modifier-dT^R, 5'-dimethoxy-trityl-5[N-(trifluoroacetylaminohexyl)-3-acrylimido]-2'-deoxyuridine, 3'-[2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite, Glen Research) within the sequence. In this case, the reactive group compatible with the cross-linker used is spaced from the 5' end or 3' end of the oligonucleotide or the modified base by at least one C2-spacer unit, but preferably by a C6-spacer unit. The nucleic acid (oligonucleotide) including a reactive linkage group then comprises at least two nucleotides.

In order to increase the stability of the nucleic acid (oligonucleotide) over cellular and extracellular nucleases, the chemically synthesized nucleic acids can be protected by a sulfurizing reagent (Beaucage-Reagenz^R, MWG- Biotech). The phosphorus diester bonds of the nucleic acid are converted into phosphorus thioate bonds in the chemical synthesis. This oligonucleotide can then be used for the enzymatic amplification of nucleic acids, extended by further linkage reactions with other nucleic acids or used directly.

In order to directly use the chimeric peptide nucleic acid fragment, the nucleic acid (oligonucleotide) should have a secondary structure that can be hybridized, preferably without internal homologies so as to be able to form a linear single-strand structure. This ensures that the nucleic acid (oligonucleotide) of the chimeric peptide-nucleic acid fragment can unfold a biochemical/therapeutic effect without further nucleic acid linkages.

However, for linkage with the signal sequence it is preferred to use nucleic acids (oligonucleotides) which have two further properties:

1. The sequence is preferably partially palindromic, has a blunt 5'-3' end ('blunt end'), an overhanging 3' end ('sticky end'), but especially has an overhanging, phosphorylated 5' end ('sticky end'), especially preferably an overhanging 5' end which comprises 4 nucleotides and has no self-homology

(palindromic sequence). As a result, a stable, monomeric secondary structure ('hairpin loop') may form. The overhanging 5' end serves for linking defined nucleic acids, antisense oligonucleotides, but preferably transcribable and replicatable genes.

2. In the apex of the 'loop', the oligonucleotide carries a modified base which carries a grouping reactive with respect to the cross-linker, preferably an amino-modified 2'-deoxythymidine. In this case, the amino function of this modified base enables the linkage reaction between MBS and oligonucleotide.

The chimeric peptide-nucleic acid fragment is suitable for appropriately introducing nucleic acids into cells and cell organelles (e.g. nucleus, chloroplast), particularly for introducing ribonucleic acids (mRNA, 'antisense' oligonucleotides) and deoxyribonucleic acids (complete gene, 'antisense' oligonucleotides). It is especially suitable for the introduction of transcribable and processable genes into mitochondria, but even more suitable for the introduction of replicative, transcription-active and processable linear-cyclic nucleic acids (plasmids).

In a preferred embodiment, a transcribable gene is linked to the nucleic acid, containing the reactive linkage site or to the chimeric peptide-nucleic acid fragment. This is effected preferably by the amplification of a gene, preferably a cloned gene consisting of a mitochondrial promoter, preferably the promoter of the light DNA strand (O_L, nt 490 - nt 369) and the gene to be expressed in a processable form, preferably a mitochondrial gene, preferably a mitochondrial transfer RNA, preferably the mitochondrial $tRNA^{Leu(UUR)}$ (nt 3204 - nt 3345) (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465). Following the enzymatic amplification of the gene, the linkage to the nucleic acid, containing the reactive linkage site, or to the chimeric peptide-nucleic acid fragment can be effected via a 'blunt end' ligation, but preferably a 'sticky end' ligation. For this purpose, the nucleic acid to be linked has at least one end capable of linkage, which consists preferably of a 5' overhang which comprises 4 nucleotides and has no self-homology (palindromic sequence). If both ends are to be linked with 'hairpin loops', a nucleic acid will preferably be selected which has differing 5'

overhangs which comprise 4 nucleotides and have no self-homology. It is especially preferred to use nucleic acids whose 5' ends also have no homology with respect to one another. For the modification of the ends (cyclization) it is then preferred to use two different 'hairpin loops', one being specific (complementary) to the 'left" plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid. In order to increase the stability of the nucleic acid over cellular and extracellular nucleases, the phosphorus diester bonds of the nucleic acid can be substituted with phosphorus thioate bonds and thus be protected if modified phosphorus thioate nucleotides have been used already in the enzymatic amplification.

A process comprising the following steps is suitable for the production of a chimeric peptide-nucleic acid fragment:

(a) Reaction of a nucleic acid (oligonucleotide), containing a functional linkage group, with a linkage agent.

(b) Reaction of the construct resulting from (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optional extension of the chimeric peptide-nucleic acid fragment resulting from (b) by further DNA or RNA fragments.

In another preferred embodiment, the chimeric peptide-nucleic acid fragment can be produced by the following steps:

(a) Optional extension of the nucleic acid, containing a functional linkage group, by further DNA or RNA fragments.

(b) Reaction of the nucleic acid with functional linkage group or the extended nucleic acid resulting from (a) with a linkage agent.

(c) Reaction of the construct resulting from (b) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL sequence.

In another embodiment which is a linear-cyclic nucleic acid in the form of a plasmid, the selection of the nucleic acid depends on the genetic information which is to be expressed in which cell and in the particular targeted compartment of the cell. In this

connection, nucleic acids which are to be transcribed have to have a suitable promoter. For example, if a gene is to be expressed in the mitochondrial matrix, a mitochondrial promoter can be chosen, preferably the promoter of the light mtDNA strand. The transcription is controlled in other cell compartments (e.g. nucleus, chloroplast) by compartment-specific promoters.

The transcription is usually regulated by what is called transcription regulation sequences, preferably mitochondrial transcription regulation sequences. In general, these sequences comprise at least binding sites for factors which initiate the transcription (transcription initiation factor) as well as the binding site for the RNA synthesis apparatus. If a transcription is to be initiated in the mitochondria, binding sequences of the mitochondrial transcription factors and of the RNA polymerase, particularly of the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase, will be suitable. In other cell compartments (e.g. nucleus, chloroplast), the transcription can be controlled by compartment-specific transcription-regulation sequences.

In order to be able to regulate the transcription, the plasmid has transcription regulation sequences which are attached preferably in the 3' direction of the transcription initiation site (promoter). For example, if the transcription of a mitochondrial transformation plasmid is to be regulated, the control elements will be suitable for the H-strand and L-strand transcription of the mitochondrial genome. However in a preferred embodiment, the control elements would be the so-called 'conserved sequence blocks' which terminate the transcription of the L-strand and simultaneously enable the transition of the DNA replication. In order to induce the exclusive transcription of the desired gene (optionally the desired genes in a polycistronic transcription), the transcription is discontinued on a suitable site behind the 3' end of the expressive gene/genes. This is achieved by the insertion of a suitable transcription-termination site, preferably arranged in the 3' direction to the promoter. For the regulated expression, the binding sequence for a bidirectionally acting transcription-termination factor is especially suitable in this case. For the transcription-termination in the mitochondria, a binding motif of a mitochondrial transcription-termination factor is preferably chosen here. At the same time, the formation

of 'antisense RNA' of the head-to-head-linked dimeric plasmids is suppressed by the use of the transcription-termination factor binding sequence.

The selection of transformed cells can be controlled via the expression of a reporter gene. Expressive genes whose expression result in a macroscopic change of the phenotype are especially suitable as reporter or selection gene. A selection is made among genes which produce resistances to antibiotics, for example. In particular, the resistance genes for oligomycin (OLI) or chloramphenicol (CAP) are suitable for the use in a mitochondrial transformation system. In this connection, the mitochondrial chloramphenicol resistance gene appears to be a particularly suitable selection gene, since CAP-sensitive cell lines already change their phenotype at a portion of about 10% of the 16 S $rRNA^{CAP+}$ gene.

The replication of the nucleic acid can be realized by an initiation site for DNA replication (replication origin). Therefore, the chimeric peptide-nucleic acid fragment in the form of a plasmid has to have at least one replication origin. In this connection, the orientation of the replication origin can be arranged irrespective of the expressive gene (genes), but preferably the replication origin is arranged in the 3' direction of the promoter. A suitable replication origin for a mitochondrial transformation plasmid would be a mitochondrial replication origin. In particular, the origin of replication of the heavy mtDNA strand is suitable in this case. It preferably has at least one "conserved sequence block". The replication can be controlled via what are called regulation sequences for the replication. For this purpose, the plasmid has to have at least one such sequence motif which is preferably arranged in the 3' direction of the promoter and the replication origin. If the replication in the mitochondria is to be regulated, a mitochondrial replication regulation sequence will be especially suitable. It is preferred to use a motif which comprises at least one of the 'termination associated sequences'. In other cell compartments (e.g. nucleus, chloroplast), the replication is initiated at least via one compartment-specific replication origin and controlled via compartment-specific replication origin and controlled via compartment-specific replication regulation sequences.

In order to permit cloning of different genes into the plasmid molecule, the plasmid nucleic acid also has to have a suitable cloning module (multiple cloning site) which has the most widely differing recognition sequences for restriction endonucleases. Here, rare

recognition sequences which do not occur on other sites of the plasmid are especially suitable. The cloning module can be incorporated into any site of the transformation plasmid. If the region of the cloning site is to be integrated into the transcription of the selection gene, the insertion of the multiple cloning site in the 3' direction of the promoter and in the 5' direction of the transcription termination site will be suitable. The integration of the multiple cloning site in the 5' direction of the selection gene is especially suitable, since in this case the use of the selection system is simultaneously accompanied by transcription of the region of the multiple cloning site.

In order to permit the autonomous replication in every targeted compartment of a cell when a nucleic acid is used, it has to be ensured that, after the synthesis of the daughter strand, the DNA replication enzymes return to the synthesis starting point again to guarantee the covalent linkage of the 3' end with the 5' end of the newly synthesized daughter strand by corresponding enzymes. For this purpose, a linear nucleic acid plasmid is suitable which can be converted into a cyclic nucleic acid. The plasmid ends can be cyclized via the use of what is called ligation-capable (phosphorylated) end of nucleic acid. For this purpose, the use of a 'blunt end' nucleic acid or a nucleic acid having overhanging 3' ends, but preferably a nucleic acid having overhanging 5' ends is particularly suitable. In this case, the overhanging ends should comprise at least one nucleotide. However, it is preferred to use overhanging 5' ends which are formed of four nucleotides. They have preferably no self-homology (palindromic sequence) and are also preferably not complementary to one another in order to suppress the formation of dimers in a subsequent nucleic acid linkage.

The cyclization of the prepared plasmid ends is arranged by synthetic oligonucleotides. They have a partial self-homology (partially palindromic sequence) and are thus capable to form what is called 'hairpin loop' structures. The partially palindromic sequence results in the formation of a stable, preferably monomeric secondary structure ('hairpin loop') having a blunt 5'-3' end (blunt end), an overhanging 3' end ('sticky end'), but preferably an overhanging 5' end. These oligonucleotides are especially preferred when they have a phosphorylated 5' end. When synthetic oligonucleotides having 'hairpin loop' structure are used, the linear plasmid DNA can be converted into a linear-cyclic system.

The ends of the two oligonucleotides are preferably complementary to one end of the prepared plasmid nucleic acid each. For this purpose, two different 'hairpin loops' are preferably used, one being specific (complementary) to the 'right' plasmid end to suppress the dimer formation. At least one of the two 'hairpin loop' oligonucleotides may have at least one modified nucleotide. It guarantees the linkage site to a signal peptide, so that the nucleic acid transport can be arranged via the protein import route. In the model case, this linkage site (modified nucleotide) is placed at one of the unpaired positions of the 'loop'. A chemically reactive group, particularly an amino or thiol function is especially suitable as linkage site.

In order to prepare the ends of the transformation plasmid of the modification (cyclization), it must be ensured that the plasmid ends are complementary to the ends of the oligonucleotides ('hairpin loops'). On the one hand, this succeeds by amplifying the plasmid DNA with suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease. In this case, recognition sequences for restriction endonucleases are suitable which do not occur repeatedly in the plasmid sequence. Especially suitable is the use of recognition sequences for restriction endonucleases generating overhanging ends ('sticky ends'), particularly those which produce overhanging 5' ends, preferably outside the own recognition sequence. In this connection, the recognition sequence for the restriction endonuclease *Bsa* I ($GGTCTCN_1N_5$) is especially suitable. On the other hand, the use of a cloned nucleic acid which already has the recognition sequences for a restriction endonuclease, preferably *Bsa* I, is suitable. As a result, the enzymatic amplification can be omitted and the nucleic acid obtained by plasmid preparation/restriction enzyme treatment can be used directly. It is preferred that the cloned nucleic acid already includes the recognition sequence for the restriction endonuclease *Bsa* I at both ends.

Various methods are available for purifying the transformation plasmid. Here, the main objective is to separate the cyclic plasmid molecule from the unreacted educts. The use of DNA-degrading enzymes are proved to be suitable in this connection. In particular, it is recommended to use enzymes which have a 5'-3' or 3'-5' exonuclease activity. Particularly the use of the exonuclease III leads to the complete hydrolysis of unreacted

educts while the cyclic plasmid DNA remains intact (no free 5' ends or 3' ends). The reaction products can be purified either via electrophoretic or chromatographic processes but also by precipitation. A selection can be made among different purification processes. On the one hand, the cyclic nucleic acid conjugated with the linkage agent and the signal peptide can be treated with an exonuclease, preferably exonuclease III, and then be purified via chromatographic electrophoretic purification and precipitation, respectively. On the other hand, the cyclic plasmid DNA can also be treated with an exonuclease, preferably exonuclease III, be purified and subsequently be conjugated with the linkage agent and the signal peptide and be purified via a chromatographic, electrophoretic purification and precipitation, respectively.

The linkage with a signal peptide can be realized by means of modified oligonucleotides. This peptide directs *in vivo* the transformation plasmid into the desired cell compartment. To this end, either the transformation plasmid can first be reacted with the modified oligonucleotide (ligation) and then the conjugation with the linkage agent and the signal peptide can take place or the modified oligonucleotide is first conjugated with the linkage agent and the signal peptide and then be used for the cyclizing the transformation plasmid ends (ligation).

The transformation system (cellular transformation) can overcome the cell membrane by various methods. Here, 'particle gun' system or microinjection are suitable, but electroporation and lipotransfection are preferred. All methods ensure the introduction of the linear-cyclic peptide nucleic acid plasmid into the cytosol of the cell from where the plasmid is directed, to its site of action (the targeted compartment) by the conjugated signal peptide.

As compared to the prior art transformation and infection methods, mentioned in the introductory part of the description, this process offers, for the first time, the possibility of appropriately introducing nucleic acids into cells and cell organelles. The selection of the signal sequence can determine the targeted compartment which is to be reached in this case (cytosol, nucleus, mitochondrion, chloroplast, etc.). Along with the compartment-specific and cell-specific introduction behavior, this process distinguishes itself by its universal applicability. Both prokaryotic and eukaryotic cells and cell systems can be treated with the

translocation vector. Since a natural transport system of the membranes is used for the appropriate introduction, the treatment of cells or cell organelles with membrane-permeabilizing agents becomes superfluous (e.g. calcium chloride method, see above).

When a replicative and transcription-active nucleic acid is used, the plasmid does not unfold its full size until the first replication cycle has been completed: As a genuine cyclic plasmid (artificial chromosome) it now has the double genetic information (head-to-head linked plasmid dimers). In particular with respect to the use of this system for a somatic gene therapy, this behavior is induced intentionally and of decisive importance, since the genes to be expressed have to compete with the defect genes of the cells. In addition to this highest possible effectiveness, the system distinguishes itself through the fact that it does not have to be integrated into a genome via a recombination step, such as retroviral systems, so as to become replicative. As a result, uncontrollable side-effects (undesired recombination) are already suppressed to the highest possible degree from the start. Therefore, the application of this plasmid system can be expected without great safety risk.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Introduction of a Chimeric Peptide-Nucleic Acid Fragment Into the Mitochondria

The overcoming of the mitochondrial double membrane system with a DNA translocation vector was studied to prove that nucleic acids can be transported appropriately

across membranes by the above-described process. For this purpose, the mitochondrial signal sequence of the ornithine transcarbamylase (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rat ornithine transcarbamylase", *Proc. Natl. Aca. Sci. U.S.A.* 80:4258-4262) (enzyme of urea cycle, naturally localized in the matrix of the mitochondria) was chemically prepared and purified. The original sequence was extended by a cysteine at the C terminus as reactive group for the subsequent linkage with the DNA (see fig. 1). This ensured that the heterobifunctional cross-linker (MBS) can only be linked with the thiol group of the only cysteine. A DNA oligonucleotide (39 nucleotides) were chosen as linkage partner. It distinguishes itself by two special features:

1. The sequence is partially palindromic and has and overhanging, phosphorylated 5' end (see fig. 1). As a result, what is called a 'hairpin loop' can form. The overhanging 5' end serves for ligating to this oligonucleotide defined nucleic acids which can then be imported into the mitochondria.
2. The oligonucleotide carries a modified base in the vertex of the 'loop' (see fig. 1). In this case, an amino-modified 2'-deoxythymidine is concerned (see fig. 2). Here, the amino function of the modified bases in this connection enables the linkage reaction between MBS and oligonucleotide.

The three reaction partners (oligonucleotide, MBS and peptide) are linked in individual reaction steps. Firstly, the oligonucleotide (50 pmoles) is reacted in a buffer (100 µl; 50 mM potassium phosphate, pH 7.6) with MBS (10 nmoles dissolved in DMSO) (reaction time: 60 min.; reaction temperature: 20°). Unreacted MBS is separated via a Nick-spin column^R, (Sephadex G 50, Pharmacia) which was equilibriated with 50 mM of potassium phosphate (pH 6.0). The eluate contains the desired reaction step with the peptide (2.5 nmoles) (reaction time: 60 min.; reaction temperature 20°C). The reaction was stopped by the addition of dithiothreitol (2 mM). The linkage product (chimera, see fig. 3) was separated via a preparative gel electrophoresis of unreacted educts and isolated from the gel by electroelution (see fig. 4). Differing nucleic acids can now be linked by simple ligation to the overhanging 5' end of the oligonucleotide.

A 283 bp long double-stranded DNA (dsDNA) was amplified via an enzymatic reaction (PCR) in the below experiment. For this purpose, a DNA fragment cloned in to

pBluescript^{R} (Stratagene) served as template DNA, which fragment in addition to the human mitochondrial promoter of the light strand (P_L, nt 902 - nt 369) included the gene for the mitochondrial transfer RNA leucine ($tRNA^{Leu(UUR)}$, nt 3204 - nt 4126) (see fig. 5). Two oligonucleotides served as amplification primers, primer 1 having a non-complementary 5' end (see fig. 5). The dsDNA was modified by the 3'-5' exonuclease activity of the T4 DNA polymerase (incubation in the presence of 1 mM dGTP) which can produce overhanging 5' ends under conditions with which a person skilled in the art is familiar (C. Aslanidis *et al.* (1990), "Ligation-independent cloning of PCR products (LIC-PCR)", *Nucleic. Acids Res.* 18: 6069-6074).

Together with the previously conjugated peptide-MBS oligonucleotide the PCR-amplified DNA could be joined using the T4 DNA ligase. In order to be able to easily detect the linkage partners after the introduction into the mitochondria, the free 5'-OH group of the ligated DNA was phosphorylated radioactively by an enzymatic reaction (A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid, I. Phosphorylation at 5'-hydroxyl termini", *J. Biol. Chem.* 241: 2923-2932; A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid. I. Further properties of the 5'-hydroxy polynucleotide kinase", *J. Biol. Chem.* 241: 2933-2943).

A fresh rat liver was comminuted for the isolation of mitochondria, suspended in 25 mM HEPES, 250 mM saccharose, 2 mM EDTA, 52 µM BSA and homogenized in a glass homogenizer (50 ml). Cell membranes, cellular debris and nuclei were centrifuged of fat 3000 g and the supernatant was prepared for another centrifugation. For this purpose, the supernatant was placed in cooled centrifuge cups and centrifuged at 8000 g. The isolation mitochondria were resuspended in 200 ml of the same buffer and centrifuged again at 8000 g. The purified mitochondria pellet was resuspended in an equal volume of the same buffer and energized by the addition of 25 mM succinate, 25 mM pyruvate and 15 mM malate. The protein content of the suspension was determined by a Bradford Testkit^{R} (Pierce). 200 µg of mitochondrial protein (energized mitochondria) were incubated together with 10 pmoles of the chimera at 37°C for 60 min. (0.6 M sorbitol, 10 mM potassium phosphate pH 7.4, 1 mM ATP, 2 mM $MgCL_2$, 1 % BSA). The mitochondria were reisolated by

centrifugation at 8000 g, resuspended in 0.6 M sorbitol, 10 mM potassium phosphate pH 7.4, 2 mM $MgCL_2$, 1 % BSA, 10 U/ml DNAse I and incubated at 37°C for 30 min. This washing step was repeated twice to remove non-specifically adhering molecules. For proving that the chimera is associated with the mitochondria, the re-isolated mitochondria were purified via sucrose gradient density centrifugation. The individual fractions of the gradient were analyzed to localize the chimera and the mitochondria. The adenylate kinase which determines cytochrome-c oxidate and malate dehydrogenase activity was used as marker for the mitochondria, while the chimera could be identified via the ^{32}p radiation measurement (see fig. 6). An analog experiment for determining the non-specific DNA introduction was carried out with the same DNA which was not linked with the signal peptide (see fig. 6). It was derived from the measurements that 65% of the chimera used segregated specifically with the mitochondria, whereas the non-specific DNA incorporation was less than 5% of the DNA used. In order to show that the chimera is not only associated with the surface of the mitochondria (membrane, import receptor), the re-isolated mitochondria were not fractioned into the three compartments of outer mitochondria membrane/intermembranous space, inner mitochondrial membrane and matrix space. For this purpose, the mitochondria were incubated with digitonin (final concentration: 1.2% w/v digitonin) and the resulting mitoplasts were separated via a sucrose gradient density centrifugation, collected in fractions and the activities of marker enzymes (adenylate kinase: intermembranous space, cytochrome c oxidase: inner mitochondrial membrane; malate dehydrogenase: matrix space) were determined according to Schnaitman and Grennawalt (C. Schnaitman *et al.* (1968), "Enzymatic properties of the inner and outer membranes of rat liver mitochondria", *J. Cell Biol.* 38: 158-175; C. Schnaitman *et al.* (1967), "The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria", *J. Cell Biol.* 32: 719-735) (see fig. 7). An analog experiment for determining the non-specific DNA incorporation was carried out with the same DNA which was not linked with the signal peptide (see fig. 7). It was derived from the measurements that 45% of the chimera are associated with the mitoplasts, whereas the non-specifically adhering DNA could be assessed to be less than 3%. The isolated mitoplasts (loss of the outer membrane and the intermembranous space) were lyzed by

Lubron^R (0.16 mg/mg protein; ICN) and separated into the compartments of inner mitochondrial membrane (pellet) and matrix space (supernatant) by ultracentrifugation at 144,000 g. The compartments were assigned via the measurement of the activities of the cytochrome c oxidase (inner mitochondrial membrane) and the malate dehydrogenase (matrix space). The chimera was measured via the detection of the ^{32}p radiation in the scintillation counter and the result was 75% segregation with the matrix of the mitochondria, while 25% of the chimera remained associated with the inner membrane of the mitochondria (incomplete translocation).

B. Example 2: Incorporation of a Replicative and Transcription-Active Chimeric Peptide-Nucleic Acid Fragment (Plasmid) Into the Mitochondria of Living Cells

In order to prove that a linear peptide-nucleic acid plasmid having cyclic ends ('hairpin loops') can overcome membranes *in vivo* via the protein import route and can be transcribed and replicated in spite of the chemical linkage with a signal peptide, the transcription and replication behavior were studies after the transfection of cells and the import into the matrix of the mitochondria. For this purpose, the signal peptide of the mitochondrial ornithine transcarbamylase was prepared synthetically, purified and linked with a nucleic acid plasmid.

Precondition for the examination of the correct transcription and replication behavior is the physical structure of the plasmid: for the experiment described below, a 3232 bp long double-stranded vector DNA (dsDNA) was cloned into pBluescript^R (Stratagene). For this purpose, the region of the mitochondrial genome was amplified via two modified oligonucleotides (primer 1, hybridized with the nucleotides 15903-15924 of the human mtDNA, includes at the 5' end an extension by the sequence TGTAGctgcag (SEQ ID NO:17) for the incorporation of a *Pst* I site; primer 2, hybridized with the nucleotides 677-657 of the human mtDNA, includes at the 5' end an extension by the sequence TTGCATGctcgagGGTCTCAGGG (SEQ ID NO:18) for the incorporation of the *Xho* I site), which comprised the promoter of the light DNA strand, the regulation motifs for the transcription (CSBs, 'conserved sequence blocks') as well as the regulation site for the DNA replication ('TAS', termination associated sequences, (D.C. Wallace (1989), "Report of the committee on human mitochondrial DNA", Cytogenet. *Cell Genet.* 51:612-

621) (see fig. 8). A multiple cloning site (MCS/TTS) was produced via a chemical synthesis of two complementary oligonucleotides (MCS/TTS 1 and 2) which contain the recognition sequences for various restriction endonucleases (see fig. 9). Under conditions with which a person skilled the art is familiar, the two oligonucleotides form hybrids which, after the phosphorylation with T4 DNA polynucleotide kinase, can be used for the ligation. In this connection, the hybrids distinguish themselves by 5' and 3' single-stranded-overhanging ends which are complementary to a *Pst* I, on the one hand, and are complementary to a *Bam* HI site, on the other hand (see fig. 9). Together with the multiple cloning site, the synthetic oligonucleotides MCS/TTS 1 and 2 also comprise a bidirectional mitochondrial transcription termination sequence (see fig. 9). It is arranged in the 3' direction of the MCS and ensures that the transcription on this site is discontinued thus correctly forming terminated transcripts forming. This sequence motif also ensures that in the cyclic plasmid system no 'antisense RNA' is expressed. The ligation reaction between pBluescript, PCR-amplified fragment and the MCS/TTS hybrids took place in a stoichiometry of 1:2:2 under conditions with which a person skilled in the art is familiar. After the transformation, several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to dideoxy sequencing (fig. 10) under conditions with which a person skilled in the art is familiar.

For the experimental examination of the replication and transcription, what is called a reporter gene was inserted in the multiple cloning site. The chloramphenicol-resistant human mitochondrial 16 S ribosomal RNA was chosen as the reporter gene. It distinguishes itself from the naturally occurring ribosomal RNA only by a modified nucleotide (polymorphism). By means of the polymerase chain reaction, a fragment having two modified ologonucleotides (primer 3, hybridized with the nucleotides 1562-1581 of the mitochondrial DNA, extended at the 5' end of the sequence CCTCTaagctt (SEQ ID NO:19) for the incorporation of a *Hind* III site; primer 4, hybridized with the nucleotides 3359-3340, extended at the 5' end of the sequence GCATTactagt (SEQ ID NO:20) for the incorporation of a *Bcl* I site) was amplified from a DNA extract of chloramphenicol-resistant HeLa cells under conditions with which a person skilled in the art is familiar. In order to ensure a correct processing of the subsequent transcript, the amplification product

included the two flanking tRNA genes ($tRNA^{Val}$ and $tRNA^{Leu}$). The amplified DNA was treated with the restriction endonucleases *Hind* II and *Bcl* I, purified by precipitation and used with the pBluescript plasmid 1 treated with *Hind* III and *Bcl* I (see figs. 8, 9 and 10) in a stoichiometry of 1:1 in a ligation reaction under conditions with which a person skilled in the art is familiar. The cloning strategy is illustrated in fig. 11.

Several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to a dideoxy sequencing under conditions with which a person skilled in the art is familiar (see fig. 12). In order to prepare the cloned DNA for the application to cell cultures and mitochondria, the cloning insert (mitochondrial transformation plasmid) was separated by the use of the restriction endonuclease *Bsa* I from the pBluescript vector under conditions with which a person skilled in the art is familiar. Alternatively, the insert DNA could be amplified via two oligonucleotides (primers 2 and 5; nucleotide sequence of primer 5: GATCCGGTCTCATTTATGCG (SEQ ID NO:21)) by the polymerase chain reaction. The use of S-dNTPs permitted the production of 'thionated' DNA which is stabilized over cellular nucleases. In both cases, the subsequent use of the restriction endonuclease *Bsa* I resulted in two different 5' overhangs. They are complementary to the 'hairpin loops' used in order to achieve a cyclization of the linear nucleic acid (see figs. 13a and b). The oligonucleotides are produced via chemical synthesis. As a result, they do not have phosphorylated 5' ends and have to be phosphorylated by a kinase reaction under conditions with which a person skilled in the art is familiar (in order to be able to subsequently examine the cellular transformation, [γ -^{32}P]-ATP was partially used in this reaction as a substrate to radioactively label the plasmid). A majority of the 'hairpin loop' structure of the oligonucleotides forms spontaneously, since the palindromic sequence can hybridize with itself. However, dimers of the 'hairpin loops' can also be converted into monomers by denaturing them in the greatest possible volume (<0.1 µM) at 93°C for at least 5 min. and fixing them immediately in a solid matrix by freezing. Then, the oligonucleotides are slowly thawed at 4°C and then 99% thereof are available in the desired monomeric 'hairpin loop' structure (see fig. 14).

The plasmid DNA was cyclized together with the two monomerized 'hairpin loops' (HP 1 and 2) in a reaction batch. In this case, the molar ratio plasmid DNA to the two 'hairpin loops' was 1:100:100 (plasmid:HP1:HP2). By using the T4 DNA ligase, the individual reactants could be combined under conditions with which a person skilled the art is familiar (see fig. 15). The ligation products were purified by a treatment with exonuclease III (reaction conditions: 37°C, 50 min.). While nucleic acids having free 3' ends are decomposed by the nuclease, the plasmid DNA linked with the two 'hairpin loops' remains stable over the 3'-5' exonuclease activity of the enzyme. The only reaction product (see fig. 15a) was separated via a preparative agarose gel electrophoresis and purified by an electroelution or by using QIAquick (Qiagen) in accordance with the manufacturer's recommendation.

The ligation product was examined via an RFLP analysis (restriction fragment length polymorphism). For this purpose, the ligated and purified plasmid DNA was treated with the restriction endonuclease *Mae* III under conditions with which a person skilled in the art is familiar. The DNA had five cleavage sites, so that fragments of differing dizes form which can be analyzed via an agarose gel (4%). Fig. 15b shows by way of example the *Mae* III cleavage pattern that is obtained after the ligation of the plasmid DNA with the two 'hairpin loops'. In this case, the DNA bands marked by the arrow tips represent the left and right end of the amplified (lane 1) and the linear-cyclic (lanes 2 and 3) mitochondrial plasmids.

For the conjugation of the circularized plasmid with the synthetic signal peptide of the rat orninthine transcarbamylase (H_2N-MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQVQ-LKPRDLC-COOH (SEQ ID NO:22)), the nucleic acid was incubated with 20 times a molar excess of m-maleimidobenzoyl-N-hydroxysuccinimide ester (linkage agent) at 20°C for 60 min. (incubation medium: 50 mM potassium phosphate pH 7.8). The excess linkage agent was separated by a 'nick spin column' (Pharmacia-LKB) under conditions with which a person skilled in the art is familiar. The 'activated' nucleic acid was conjugated by reacting the nucleic acid with 50 times the molar excess of the signal peptide at 20°C (incubation medium: 50 mM potassium phosphate pH 7.8). The reaction was stopped by the addition

of 1 mM dithiothreitol after 45 min. and the conjugate was available for the experiments to come.

In order to be able to show the *in vivo* usability of the peptide-nucleic acid plasmid, the plasmid had to be incorporated into eukaryotic cells. For this purpose, a chloramphenicol-sensitive B lymphocyte or fibroblast cell culture was transfected via a lipotransfection with the peptide-nucleic acid plasmid (the labeling was introduced at ^{32}p labeling during the kinase reaction of the 'hairpin loop' (HP1)) was pre-incubated together with 2-6 µl serum-free Optimem^R (Gibco-BRL) (20°C, 15 min.). During the incubation the polycationic lipid of the LipofectAmine^R reagent DOSPA (2,3-dioleyloxy-N-[2-(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate) forms unillamellar liposomes with the aid of the neutral lipid DOPE (dioleoylphosphatidylethanolamine), which can complex the DNA. Then, the reaction batch was added to the prepared cells, adjusted to a density of about $2.5*10^6$ cells per 0.8 ml (35 mm culture dishes, 4 h, 37°C, CO_2 incubator). The transfection medium was then replaced by 5 ml of DMEM medium (Gibco-BRL) previously supplemented by 10% fetal calf serum and 100 µg/ml chloramphenicol. The transformation efficiency was determined by the measurement of the ^{32}P radiation of the construct. As a rule, a cellular incorporation rate of 80-85% of the chimeric construct were associated with the transformed cells and 15-20% of the chimeric peptide-DNA plasmid remained in the supernatant of the transfection reaction.

After about 21-28 days, chloramphenicol-resistant colonies formed in the transformed cells. Under conditions with which a person skilled in the art is familiar, the resistant cells were isolated and multiplied. Under conditions with which a person skilled in the art is familiar, sufficient DNA could be obtained from about $1*10^5$ cells to classify the genotypes. For this purpose, the isolated DNA was separated via agarose gel electrophoresis and transmitted to a nylon membrane (Southern blot). The nucleic acids were detected by hybridization using a specific, radioactively labeled probe (see fig. 16). In addition to the introduced circularized 'linear' vector (lanes 2 and 6) an 'in vitro' transcription (lane 3), an 'in vitro' replication (lane 4), as well as the intermediates obtained 'in vivo' (isolated nucleic acids of a transformed clone) are shown in this illustration.

While the three smaller bands can be produced in vitro by incubating the circularized vector with the four nucleoside triphosphates (RNA) and a mitochondrial enzyme extract (lane 3), the formation of a dimer, circular plasmid (greatest band in lane 4) is observed in the further addition of the deoxynucleoside triphosphates to the reaction batch: an identical image yields the analysis of the nucleic acids which can be obtained from transformed cell colonies (lane 5). The fact that the greatest DNA band in lanes 4 and 5 is actually the dimeric and thus replicated mitochondrial plasmid, could be confirmed by sequence analysis.

A lipotransfection batch where the non-conjugated plasmid not linked with the signal peptide was used, served as a control experiment. As expected, this plasmid was not incorporated into the mitochondria of the transfected cells and thus did not result in the formation of chloramphenicol-resistant cells. These cells stopped growth after 10 days and decayed within the following 8 to 10 days completely.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED:

1. A chimerical peptide-nucleic acid fragment comprising:

   (a) a cell-specific, compartment-specific or membrane-specific signal peptide, with the exception of a KDEL signal sequence,

   (b) a linkage agent,

   (c) a nucleic acid (oligonucleotide),

the signal peptide being linked via the linkage agent which via amino acids at the carboxy-terminal end of the signal peptide is linked therewith so as to ensure the appropriate nucleic acid introduction into cell organelles and cells.

2. The chimerical peptide-nucleic acid fragment according to claim 1, characterized in that the nucleic acid consists of at least two bases.

3. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 2, characterized in that the nucleic acid has a secondary structure.

4. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 3, characterized in that the nucleic acid has a palindromic sequence.

5. The chimerical peptide-nucleic acid fragment according to claim 4, characterized in that the nucleic acid may form a "hairpin loop".

6. The chimerical peptide-nucleic acid fragment according to claim 5, characterized in that the nucleic acid may hybridize with itself and may form an overhanging 3' end or 5' end ('sticky end').

7. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 6, characterized in that the nucleic acid is a ribonucleic acid, preferably a deoxyribonucleic acid.

8. The chimerical peptide-nucleic acid fragment according to claim 7, characterized in that the nucleic acid has chemically modified 'phosphorous thioate' linkages.

9. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 8, characterized in that the nucleic acid carries a reactive linkage group.

10. The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains an amino function when the linkage agent contains an amino-reactive grouping.

11. The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains a thiol function when the linkage agent contains a thiol-reactive grouping.

12. The chimerical peptide-nucleic acid fragment according to claim 10 or 11, characterized in that the linkage grouping present is bound to the nucleic acid via at least one C2 spacer, but preferably one C6 spacer.

13. The chimerical peptide-nucleic acid fragment according to claim 12, characterized in that the linkage grouping is localized at the 3' hydroxy/phosphate terminus or at the 5' hydroxy/phosphate terminus of the nucleic acid, but preferably at the base.

14. The chimerical peptide-nucleic acid fragment according to any one of claims 10 to 13, characterized in that defined nucleic acids, antisense oligonucleotides, messenger RNAs or transcribable and/or replicatable genes are linked with the 5' end and/or 3' end.

15. The chimerical peptide-nucleic acid fragment according to claim 14, characterized in that the nucleic acid to be linked contains chemically modified 'phosphorus thioate' linkages.

16. The chimerical peptide-nucleic acid fragment according to claim 14 to 15, characterized in that the gene be linked contains a promotor, preferably a mitochondrial promoter.

17. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 16, characterized in that the signal peptide has a reactive amino acid at the carboxy-terminal end, preferably a lysine or cysteine, when the linkage agent contains an amino-reactive or thiol-reactive grouping.

18. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 17, characterized in that the signal peptide carries a cell-specific, compartment-specific or membrane-specific recognition signal.

19. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 18, characterized in that the signal peptide has a cell-specific, compartment-specific or membrane-specific peptidase cleavage site.

20. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 19, characterized in that the peptide consists of the compartment-specific cleavable signal peptide of the human mitochondrial ornithine transcarbamylase, extended by an artificial cysteine at the C terminus.

21. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 20, characterized in that the linkage agent is a bifunctional, preferably heterobifunctional cross-linker.

22. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 21, characterized in that the linkage agent contains thiol-reactive and/or amino-reactive groupings when the signal peptide and the nucleic acid carry thiol and/or amino groups as linkage sites.

23. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 22, characterized in that the linkage agent is m-maleimido-benzoyl-N-hydroxy-succinimide ester or a derivative thereof.

24. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 23, characterized in that the molecule can overcome membranes with and without membrane potential by utilizing natural transport mechanisms.

25. The chimerical peptide-nucleic acid fragment in the form of a linear-cyclic plasmid, characterized in that the plasmid comprises at least one replication origin and that both ends of the nucleic acid portion are cyclized, at least one cyclic end having a modified nucleotide which via a linkage agent can be liked with a cell-specific, compartment-specific or membrane-specific signal peptide.

26. The chimerical peptide-nucleic acid fragment according to claim 25, characterized in that the nucleic acid portion further comprises at least one promoter, preferably a mitochondrial promoter, especially preferably the mitochondrial promoter of the light strand.

27. The chimerical peptide-nucleic acid fragment according to any one of claims 25 and 26, characterized in that the nucleic acid portion further comprises transcription-regulatory sequences, preferably mitochondrial transcription-regulatory sequences.

28. The chimerical peptide-nucleic acid fragment according to any one of Claims 25-27, characterized in that the transcription-regulatory sequences have at least one binding site of a transcription initiation factor.

29. The chimerical peptide-nucleic acid fragment according to any one of Claims 25 to 28, characterized in that the transcription-regulatory sequences have at least one binding site

for the RNA synthesis apparatus, preferably the binding site for the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase.

30. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 29, characterized in that the transcription-regulatory sequences are arranged in the 3' direction of the promoter.

31. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 30, characterized in that the transcription is regulated by elements of the mitochondrial H-strand and L-strand transcription control.

32. The chimerical peptide-nucleic acid fragment according to claim 31, characterized in that what is called 'conserved-sequence-blocks' of L-strand transcription act as transcription control elements.

33. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 32, characterized in that the plasmid further comprises at least one transcription termination site.

34. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 33, characterized in that the transcription termination site has a binding sequence of a mitochondrial transcription termination factor.

35. The chimerical peptide-nucleic acid fragment according to claim 34, characterized in that the transcription termination site has the binding sequence of a preferably bidirectionally acting transcription termination factor.

36. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 35, characterized in that the replication origin is a mitochondrial replication origin,

preferably the replication origin of the heavy mtDNA strand having at least one 'conserved sequence block'.

37. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 36, characterized in that the plasmid further comprises at least one regulatory sequence for the replication.

38. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 37, characterized in that the regulatory sequence for the replication is a mitochondrial sequence motif.

39. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 38, characterized in that the plasmid further comprises a selection gene, preferably an antibiotic-resistance gene, preferably the oligomycin - or chloramphenicol - resistance gene.

40. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 39, characterized in that the plasmid further contains a multiple cloning site which permits the expression of 'foreign genes'.

41. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 40, characterized in that the multiple cloning site comprises recognition sequences for restriction endonucleases which do preferably not occur in another site of the plasmid.

42. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 41, characterized in that the multiple cloning site is arranged in the 3' direction of the promoter and in the 5' direction of the transcription termination site.

43. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 42, characterized in that the multiple cloning site is arranged in the 5' direction of the selection gene.

44. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 43, characterized in that the nucleic acid fragment has (phosphorylated) ends capable of litigation.

45. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 44, characterized in that the nucleic acid fragment has `blunt ends' or overhanging 3' ends, preferably overhanging 5' ends.

46. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 45, characterized in that the nucleic acid fragment has 4 nucleotides comprising 5' overhangs which do not have a self-homology (palindromic sequence) and are not complementary to one another either.

47. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 46, characterized in that the ends of the nucleic acid fragment are cyclized via synthetic oligonucleotides.

48. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 47, characterized in that the overhanging 5' ends of the two oligonucleotides are complementary to one differing end of the nucleic acid each.

49. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 48, characterized in that two differing `hairpin loops' are used for the cyclization, one being specific (complementary) to the `left' plasmid end and the other being specific to the `right' plasmid end of the nucleic acid.

50. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 49, characterized in that the modified nucleotide is localized preferably within the `loop'.

51. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 50, characterized in that the plasmid DNA is amplified enzymatically by suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease which occurs preferably in non-repeated fashion in the plasmid sequence.

52. The chimerical peptide-nucleic acid fragment according to claim 51, characterized in that the restriction endonuclease to be used generated overhanging ends, preferably 5' overhanging ends, the cleavage site being localized preferably outside the recognition sequence.

53. The chimerical peptide-nucleic acid fragment according to claim 51 or 52, characterized in that the restriction endonuclease is *BsaI*.

54. A process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following stages:

(a) reaction of a nucleic acid (oligonucleotide) containing a functional linkage group having a linkage agent,

(b) reaction of the construct of (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optionally extension of the chimerical peptide-nucleic acid fragment of (b) by further DNA or RNA fragments.

55. The process according to claim 54, characterized in that the DNA in step (c) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR}$).

56. The process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following steps:

(a) optional extension of the nucleic acid containing a functional linkage group by further DNA or RNA fragments,

(b) reaction of the nucleic acid with functional linkage group or the extended nucleic acid of (a) with a linkage agent,

(c) reaction of the construct of (b) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence.

57. The process according to claim 56, characterized in that the DNA in step (a) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine ($tRNALeu^{UUR}$).

58. Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53 for the appropriate nucleic acid introduction into cell organelles and cells, characterized by reacting the fragment with cells or pretreated cell compartments.

59. Use according to claim 58, characterized in that the pretreated cell compartments are energized mitochondria.

60. Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 59 for the introduction into eukaryotic cells.

61. Use of a chimerical peptide-nucleic acid fragment according to claim 60, characterized by employing the 'particle gun' system, electroporation, microinjection or lipotransfection for the introduction into eukaryotic cells.

ABSTRACT

This invention relates to a chimeric peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

CHIMERIC PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USE FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

Inventors: **Peter Seibel**
Andrea Seibel

Albert P. Halluin, Esq.
PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711

(415) 854-3660

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

CHIMERIC PEPTIDE-NUCLEIC ACID FRAGMENT, PROCESS FOR PRODUCING THE SAME AND ITS USE FOR APPROPRIATELY INTRODUCING NUCLEIC ACIDS INTO CELL ORGANELLES AND CELLS

This is a national phase filing of the Application No. PCT/DE95/00775, which was filed with the Patent Corporation Treaty on June 11, 1995, and is entitled to priority of the German Patent Application P 44 21 079.5, filed June 16, 1994.

I. FIELD OF THE INVENTION

[Chimerical peptide-nucleic acid fragment, process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.]

This invention relates to a [chimerical] chimeric peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

II. BACKGROUND OF THE INVENTION

It is known that cellular membrane systems are largely impermeable to nucleic acids. However, cell membranes can be [overcome] penetrated very efficiently by physical processes (transformation) and biological processes (infection).

Transformation, i.e., the [direct] direction absorption of [the] naked nucleic acid by [the cell] cells, is preceded by cell treatment. There are various methods available for the production of these ['competent cells']"competent cells". Most processes are based on the observations made by Mandel and Higa (M. Mandel *et al.,* (1970), "Calcium-dependent bacteriophage DNA infection", *J. Mol. Biol.* 53: 159-162), who [could] managed to show for the first time that [the] yields resulting from the absorption of [$\lambda\alpha\mu\beta\delta\alpha$] λ-DNA by bacteria can be increased [fundamentally] significantly in the presence of calcium chloride. This method [is] was also used successfully for the first time by Cohen *et al.* (S.N. Cohen *et al.* (1972), "Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA", *Proc. Natl. Acad. Sci. U.S.A.* 69:2110-2114) for

plasmid DNA and was improved by many modifications (M. Dagert *et al.* (1979), "Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells", *Gene* 6:23-28). Another transformation method is based on the observation that high-frequency alternating fields may break up cell membranes (electroporation). This technique can not only be used to introduce naked DNA into [not only] prokaryotic cells but also eukaryotic cell systems (K. Shigekawa *et al.* (1988), "Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells", *Biotechniques* 6:742-751). Two very gentle methods of introducing DNA into eukaryotic cells were developed by Capecchi (M.R. Capecchi (1980)), "High efficiency transformation by direct microinjection of DNA into cultured mammalian cells" *Cell* 22:479-488) and Klein *et al.* (T.M. Klein *et al.* (1987), "High velocity microprojectiles for delivering nucleic acids into living cells", *Nature* 327:70-73)[:]. They are based, respectively, on the direct injection of the DNA into the individual cell (microinjection), [on the one hand,] and on the bombardment of a cell population with microprojectiles consisting of tungsten, to the surface of which the corresponding nucleic acid [was] is bound ('shotgun').

_____The biological infection methods have proved their value [parallel to] concurrently with the physical transformation of cells. [They] In particular, they include [particularly] the highly efficient viral introduction of nucleic acids into cells (K.L. Berkner (1988), "Development of adenovirus vectors for the expression of heterologous genes", *Biotechniques* 6:616-629; L.K. Miller (1989), "Insect baculoviruses: powerful gene expression vectors", *Bioessays* 11:91-95; B. Moss *et al.* [(1990)](199), "Product review. New mammalian expression vectors", *Nature* 348:91-92) and the liposome mediated lipofection (R.J. Mannino *et al.* (1988), "Liposome mediated gene transfer", *Biotechniques* 6:682-690; P.L. Felgner *et al.* (1987), "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure", *Proc. Natl. Acad. Sci. U.S.A.* 84:7413-7417).

_____All methods described so far deal with [the] overcoming [of] the prokaryotic or eukaryotic plasma membrane by naked or packaged nucleic acids. While the site of action is reached [already] immediately when the nucleic [acid] acids are introduced into [the] a prokaryotic cell, further biochemical processes take place in a compartmentalized eukaryotic cell, which [support] allow the penetration of the nucleic acid into the nucleus

under certain conditions (e.g., viral route of infection in the case of HIV). Analogous infective processes in which exogenous nucleic acids are actively introduced into other cell organelles (e.g., into mitochondria) have not been described so far.

In addition to the introduction of the nucleic acid into the cell and cell organelle, respectively, the transcription and [above all] particularly the replication of the introduced nucleic acid play a decisive [part] role. In this connection, it is known that [the] DNA molecules may have a special property which permits duplication in a cell under certain conditions. A special structural element, the origin of the DNA replication [(ori, origin)](also known as "ori", or "origin"), adds thereto. Its presence provides the ability of DNA replication (K.J. Marians (1992), "Prokaryotic DNA replication", *Annu. Rev. Biochem.* 61:673-719; M.L. DePamphilis (1993), "[Eukaryotic] Eurkaryotic DNA replication: anatomy of an origin", *Annu. Rev. Biochem.* 62:29-63; H. Echols and M.F. Goodman (1991), "Fidelity mechanisms in DNA replication", *Annu. Rev. Biochem.* 60:477-511). The strictly controlled process of DNA replication starts in E. coli e.g., when a protein is bound (K. Geider and H. [Hoffmann] Hoffman Berling (1981), "Proteins controlling the helical structure of DNA", *Annu. Rev. Biochem.* 50:233-260) to the highly specific initiation site thus defining the starting point of a specific RNA polymerase (primase). It synthesizes a short RNA strand [(~10)(~10 nucleotides, 'primer') which is complementary to one of the DNA template strands. The 3' hydroxyl group of the terminal ribonucleotide of this RNA chain serves as ['primer']a "primer" for the synthesis of new DNA by a DNA polymerase. DNA-untwisting proteins unwind the DNA double helix (J.C. Wang (1985), "DNA topoisomerases", Annu. Rev. Biochem. 54:665-697). The separated individual strands are stabilized by DNA-binding proteins as regards their conformation (J.W. Chase and K.R. Williams (1986), "Single-stranded DNA binding proteins required for DNA replication", *Annu. Rev. Biochem.* 55:103-136) to enable proper functioning of the DNA polymerases (T.S. Wang (1991), "Eukaryotic DNA polymerases", *Annu. Rev. Biochem.* 60:513-552). A multienzyme complex, the holoenzyme of DNA-polymerase-III, synthesizes the majority of the new DNA. The RNA portion of the [chimerical] chimeric RNA-DNA molecule is then split off the DNA polymerase III. The removal of the RNA from the newly formed DNA chains creates gaps between the DNA fragments[.] These

gaps are filled by the DNA-polymerase I which can newly synthesize DNA from a single-stranded template. While one of the two newly synthesized DNA strands is synthesized continuously (5'-3' direction, leader strand), Ogawa and Okazaki observed that a majority of the newly synthesized opposite strand (3'-5' direction, delayed strand) was synthesized from short DNA fragments (T. Ogawa and T. Okazaki (1980), "Discontinuous DNA replication", *Annu. Rev. Biochem.* 49:421-457). Here, what [is] are called primases initiate the onset of the DNA synthesis of the opposite strand by the synthesis of several RNA primers. When the replication proceeds, these fragments are freed from their RNA primers, the gaps are closed and covalently linked with one another to give extended daughter strands by the DNA ligase. Two chromosomes form after the termination of the replication cycle.

As opposed thereto, the DNA replication is controlled by many plasmids via a replication origin which dispenses with the synthesis of the delayed strand (3'-5' direction) and can initiate the synthesis of two continuous DNA strands bidirectionally (each in the 5'-3' direction along the two templates). The precondition for [a] complete DNA replication [is] here is the cyclic form of the nucleic acid. It ensures that at the end of the new synthesis of the complementary DNA strands the DNA polymerases return to the starting point again where [now] ligases now guarantee the covalent linkage of the ends of the two newly synthesized daughter strands.

Smallpox viruses represent an interesting form of linear-cyclic nucleic acids: because of what is called ['hairpin loops']"hairpin loops" at the ends of their linear genomes [they] their molecules have a cyclic [molecule] structure while maintaining a predominantly linear conformation (D.N. Black *et al.* (1986), "Genomic relationship between capripoxviruses", *Virus Res.* 5:277-292; J.J. Esposito and J.C. Knight (1985) "[Orthopoxvirus] Orthopoxyvirus DNA: a comparison of restriction profiles and maps", *Virology* 143:230-251). Covalently closed ['hairpin]"hairpin" nucleic acids were not only found in smallpox viruses but also described for the ribosomal RNA from Tetrahymena (E.H. Blackburn and [J.G] J.T. Gall (1978), "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena", *J. Mol. Biol.* 120:33-53) and the genomes of the parvoviruses (S.E. Straus *et al.* (1976), "Concatemers of alternating

plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis", *Proc. Natl. Acad. Sci. U.S.A.* 73:742-746; P. Tattersall and D.C. Ward (1976), "Rolling hairpin model for the replication of parvovirus and linear chromosomal DNA", *Nature* 263:106-109).

However, [by means of the formerly known plasmids or nucleic acid constructs it is not possible to appropriately introduce nucleic acids into cells or cell organelles via the protein import route. But this is e.g. a precondition for treating genetically changes of the mitochondrial genomes of patients suffering from neuromuscular and neurodegenerative diseases or carrying out an appropriate mutagenesis in mitochondria or other cell organelles.

Therefore, it was] it is not possible to introduce nucleic acids into cells or cell organelles adequately using the protein import route by means of the formerly known plasmids or nucleic acid constructs. But such an approach is, for example, a precondition for treating genetic changes for the mitochondrial genomes of patients suffering from neuromuscular and neurodegenerative diseases or for carrying out an appropriate mutagenesis in mitochondria or other cell organelles.

III. SUMMARY OF THE INVENTION

This invention relates to a chimeric peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is explained particularly the figures, wherein:

Figure 1 depicts a signal peptide of the ornithine transcarbamylase of rats as well as a DNA sequence suitable for introduction into a cell organelle. Top: signal peptide of the ornithine transcarbamylase of rats (32 amino acids), extended by 10 N-terminal amino acids of the matured protein and an additional cysteine as linkage site (SEQ ID NO:1). The peptide sequence is shown in the international one-letter code; middle: a partially palindromic DNA sequence suitable for introduction and consisting of 39 nucleotides

having an amino-modified T at nucleotide position 22 (SEQ ID NO:2); bottom: marked secondary structure of the oligonucleotide having an overhanging 5' end and a modified nucleotide in the vertex of the 'loop'.

Figure 2 depicts the structure of the amino-modified 2-deoxythymidine, R: nucleic acid residues.

Figure 3 depicts a diagram of chimeric peptide-nucleic acid fragment, consisting of amino-modified oligonucleotide (39 nucleotides) with marked 'hairpin loop', cross-linker and signal peptide. CL: Cross=linker.

Figure 4 the electrophoretic separation of the linkage product resulting from amino-modified oligonucleotide (39 nucleotides), m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) and signal peptide of the ornithine transcarbamylase of rats (42 amino acids, extended by a cysteine at the C terminus).

Figure 5A depicts a flow diagram of the peptide-DNA fusion, cloning, amplification and linkage of the transcribable and processable mitochondrial tRNA gene to be introduced (S. Anderson et al. (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465) (SEQ ID NOS:2-6). CL: cross-linker (MBS); MCS: multiple cloning site of pBluescript^{R} (Stratagene), mtTF: binding site of the mitochondrial transcription factor; RNA-Pol: binding site of the mitochondrial RNA polymerase; tRNA Leucin: gene of the mitochondrial transfer RNA for leucine (UUR); *Sac II, Apa I, Eco RI*: sites for restriction endonucleases; the cloned mitochondrial sequences were numbered in accordance with the published sequence of the human mitochondrial genome (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465)

Figure 5B and 5C depicts the sequence of the cloned $tRNA^{Leu(UUR)}$ gene (SEQ ID NOS:7 and 8).

Figure 6A and 6B depict a representation of the ^{32}P radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitochondria-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total

radiation/enzyme activity which was plotted against the gradient is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 7A and 7B depicts a representation of the ^{32}P radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitoplast-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient, is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Figure 8 depicts the cloning of the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 1). Using the two oligonucleotides (primers 1 and 2), the gene section of nucleotide 15903 to nucleotide 677 was amplified enzymatically from mitochondrial HeLa DNA (comprises: promoter characterized by the binding sites for the mitochondrial transcription factors and the RNA polymerase; replication origin characterized by what is called 'conserved sequence blocks'; regulation of the DNA replication characterized by the 'TAS' motifs). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Xho* I and *Pst* I, the ends of the amplified nucleic acid can be modified such that they are compatible with a vector arm of pBluescript, on the one hand, and compatible with the hybrid of the oligonucleotides MCS/TTS 1 and 2, on the other hand. In addition to a multiple cloning site, they also comprise a transcription termination sequence which is responsible for the regulated transcription. The ligation product is then transformed into *E. coli* XL 1. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to RFLP and sequence analysis.

Figure 9 depicts the sequence of the oligonucleotides MCS/TTS 1 and 2 (SEQ ID NOS:9 and 10). The oligonucleotides MCS 1 and 2 were prepared synthetically and comprise recognition sequences for nine different restriction endonucleases as well as a sequence motif which can suppress the transcription bidirectionally. The olignucleotides are complementary and can thus form a hybrid. The overhanging ends are part of the recognition sequences for the restriction endonucleases *PstI* and *Bam* HI.

Figure 10 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 1) (SEQ ID NOS:11 and 12).

Figure 11 depicts the cloning of the reporter gene into the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 2). Using the two oligonucleotides (primers 3 and 4), the gene section of nucleotide 1562 to nucleotide 3359 was amplified enzymatically from a DNA extract of a human CAP=resistant cell line (comprises: part of the 12 S rRNA gene, $tRNV^{Val}$ gene, 16 S $rRNA^{CAP+}$ gene, $tRNA^{Leu}$ gene, part of ND 1 gene). Since the oligonucleotides contain recognition sequences for the restriction endonucleases *Hind* III and *Bcl* I, the ends of the amplified nucleic acid can be modified such that they are compatible with the multiple cloning site (MCS) of the peptide-nucleic acid plasmid (plasmid 1). The ligation product is then transformed in *E. coli* XL 1 Blue. Following the plasmid isolation of insert-carrying *E. coli* colonies, the nucleic acids were subjected to the RFLP and sequence analysis and are available for the described experiments.

Figure 12 depicts the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid including the reporter gene (plasmid 2) (SEQ ID NOS:13 and 14).

Figure 13A depicts the reaction run of the cyclization of the nucleic acid portion as well as the conjugation of the nucleic acid portion with a signal peptide. The nucleic acid portion of the peptide-nucleic acid plasmid can be obtained via a plasmid preparation or an enzymatic amplification. In both cases, the treatment with the restriction endonuclease *Bsa* I results in an intermediate product capable of ligation. It can be reacted directly with the monomerized 'hairpin loops'. The reaction product is freed by an exonuclease III treatment from non-specific (non-cyclic) reaction products and educts, is purified and conjugated with the signal peptide via a cross-linker. As an alternative, one of the two 'hairpin loops' can first be conjugated with the signal peptide via a cross-linker before the cyclizing ligation reaction is carried out. A purification of the reaction product follows an exonuclease III treatment here as well.

Figure 13B depicts the structure and sequence of the 'hairpin loop' oligonucleotides HP 1 (SEQ ID NO:2) and 2 (SEQ ID NO:15).

V Figure 14 depicts the monomerization of a 'hairpin loop' oligonucleotide. The synthetic 'hairpin loops' HP 1 and 2 can be monomerized by a thermal or alkaline denaturation. This figure shows a standard agarose gel: lane 1, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III), lane 2: HP 1, synthesis product; lane 3: HP 1, thermally monomerized.

Figure 15 depicts a ligation reaction between the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 2) and the 'hairpin loops' HP 1 and 2. This figure shows a standard agarose gel: lane 1, cloned nucleic acid portion of the peptide-nucleic acid portion in pBluescript treated with the restriction endonuclease *Bsa* I, lane 2: ligation of the reaction products resulting from lane 1 with the 'hairpin loops' HP 1 and 2; lane 3, treatment of the reaction production resulting from lane 2 with exonuclease III; lane 4, molecular weight standard (λDNA treated with the restriction endonucleases *HIND* III and *Eco* RI).

Figure 15B depicts the examination of the purified ligation product by a *Mae* III-RFLP analysis. This figure illustrates a standard agarose gel: lane 1, enzymatically amplified nucleic acid portion following a *Mae III* treatment; lane 2: purified ligation product of the enzymatically amplified nucleic acid portion following a *Mae* III treatment; lane 3: purified product of the plasmid DNA ligation following a *Mae* III treatment; lane 4, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease *Hae* III).

Figure 16 depicts the transcription and replication of the peptide-nucleic acid plasmid. This figure illustrates a standard agarose gel: lane 1, molecular weight standard (λDNA treated with the restriction endonucleases *Hind* III and *Eco* RI); lane 2, untreated peptide-nucleic acid plasmid; lane 3: *in vitro*-obtained transcription products of the peptide-nucleic acid plasmid; lane 4: *in vitro*-obtained replication and transcription products of the peptide-nucleic acid plasmid; lane 5, *in vivo*-obtained replication and transcription products of the peptide nucleic acid plasmid; lane 6, untreated peptide-nucleic acid plasmid.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to develop a construct on a nucleic acid basis which permits the appropriate introduction of nucleic acids into cell and compartments of eukaryotic cells. Furthermore, a process is to be provided of how this construct can reach cell compartments or cells. In addition, the introduced nucleic acid should be such that it can also be incorporated as replicative nucleic acid via cellular protein import routes. [Besides] Additionally, properties should be present which result in a controlled transcription and/or replication in cells and in defined [aimed] targeted compartments of cell, respectively. The process is to be used for the therapy of genetic diseases (changes of the mitochondrial genome) [ad] and for the appropriate mutagenesis in eukaryotic and prokaryotic cells. The invention is intended to meet the following demands:

- universal applicability;
- cell-specific, compartment-specific and membrane-specific introduction [behavior] capability;
- high degree of effectiveness;
- low immunogenicity;
- minimization of the infection risk;
- the introduced nucleic acid (plasmid molecule) is to be replicatable;
- the introduced nucleic acid [(plasmic](plasmid molecule) is to be transcribable;
- the introduced nucleic acid (plasmid molecule) shall be resistant to nucleases; and
- the structure of the introduced nucleic acid (plasmid molecule) should be universally usable.

This problem is solved by the features of claims 1), 25), 54), 56), 58), 60) and 61). Advantageous embodiments follow from the subclaims.

In order to be able to appropriately carry a protein within a cell from the site of formation to another compartment or another cell organelle (e.g. the site of action), the protein is usually synthesized as a preprotein (R. Zimmermann et al. (1983), "Biosynthesis and assembly of nuclear-coded mitochondrial membrane proteins in Neurospora crassa", *Methods Enzymol.* 97:275-286). In addition to the matured amino acid sequence, the

preprotein has what is called a signal sequence. This signal sequence is specific to the [aimed] targeted compartment and enables [that he] the preprotein [can] to be recognized by surface receptors. The natural obstacle ['membrane']that the "membrane" presents is then overcome by translocating the preprotein through the membrane by an active [(]process (in which several 'transport proteins' are involved [in this process) or]) or a passive process [(](i.e., direct passage without involvement of further proteins). Thereafter, the signal sequence is usually separated on the site of action by a specific peptidase unless it is a constituent of the matured protein. The matured protein can now unfold its enzymatic activity.

The inventors have recognized that this mechanism can appropriately be utilized to [appropriately] transport nucleic acids across membranes. In this case, the nucleic acid is not subject to a restriction, i.e., it is possible to use every nucleic acid desired and known, respectively. For this purpose, a cell-specific, compartment-specific or membrane-specific signal sequence is linked with the desired nucleic acid, resulting in a [chimerical] chimeric peptide-nucleic acid fragment. In this context, it is known that the linkage between a nucleic acid and a peptide may occur via the α-amino group of a synthetic KDEL (SEQ ID NO:16) peptide, modified by ϵ-maleimidocapronic acid-N-hydroxysuccinimide ester (K. Arar *et al.* (1993), "Synthesis of oligonucleotide-peptide conjugates containing a KDEL signal sequence", *Tetrahedron Lett.* 34:8087-8090). However, this linkage strategy is completely unusable for the [nucleic] introduction of nucleic acids into cell organelles and cells, since here the translocation should occur in analogy to the natural [protein] transport of proteins. Such a transport cannot be expected by blocking the α-amino group of a synthetic peptide by means of a nucleic acid. Therefore, the inventors chose linkage via a carboxy-terminal amino acid. On the one hand, this ensures a 'linear' linkage, on the other hand, the free amino-terminal end of the signal peptide is thus available for the essential steps of the import reaction.

In order to be able to utilize the described transport mechanism also for the introduction of replicative and transcription-active nucleic acids, the nucleic acid is preferably integrated via a homologous recombination into an existing genome or is itself the carrier of the genetic elements, which ensures an autonomous initiation of replication

and transcription. Only the latter variant complies with the criterion of universal applicability, since a recombination into an existing cellular genome is successful only under certain conditions and in select cells.

In this case, the use of cyclic DNA represents one possibility, since the DNA polymerases at the end of the new synthesis of the daughter strands return to the initial point thus guaranteeing a complete DNA replication. Although the use of a double-stranded cyclic plasmid meets all physical criteria for a successful replication in every [aimed] targeted compartment of the cell, this physical DNA form is confronted with the import pore size which is decisively involved in the appropriate translocation: Even the compact diameter of a superhelical plasmid can be compared with that of globular proteins, therefore, a translocation through a membrane system via the protein import route appears impossible. Here, an approach to a solution [consists in] involves the use of linear-cyclic DNA molecules having modified (cyclic) ends but [only the] whose diameter is only the size of linear DNA molecules. On the one hand, they are [no obstacle for] not too large to go through the import pore [size]; on the other hand, these linear-cyclic DNA molecules include all physical preconditions to be able to form replicative and transcription-active plasmids in the mitochondria.

The following is preferably required for the construction of the [chimerical] chimeric peptide-nucleic acid fragment according to the invention as well as for the construction of a replicative and transcription-active nucleic acid portion (plasmid):

- signal peptide and signal sequence, respectively, (cell-specific, compartment-specific, or membrane-specific);
- linkage agent; and
- nucleic acid (oligonucleotide) which may preferably comprise the following [further] additional information:
  - information [on] in the initiation and regulation of transcription and replication,
  - information as to the termination of transcription and replication,

- multiple cloning site for a nucleic acid to be introduced (to be expressed) additionally,
- possible modifications, so that 'hairpin loops' can be added (cyclization of the ends) which permit linkage with the signal peptide.

The selection of the signal sequence depends on the membrane and membrane system, respectively, which is to be overcome and the [aimed] targeted compartment of the cell (cell nucleus, mitochondrion, chloroplast) or the cell organelle which is to be obtained. Proteins which are to be introduced, e.g., into one of the four mitochondrial compartments (outer mitochondrial membrane, intermembraneous space, inner mitochondrial membrane, matrix space), have compartment-specific signal sequences. In general, signal sequences are chosen for the introduction of nucleic acids which contain a cell [-]specific, compartment-specific or membrane-specific recognition signal thus directing the attached nucleic acid [to] of its site of action (e.g., inner side of the inner mitochondrial membrane or matrix space). A selection can be made among signal sequences which can transport proteins in the presence or absence of a membrane potential.

_____For the introduction of nucleic acid [introduction], signal sequences which function irrespective of the membrane potential are preferred, e.g., the signal sequence of ornithine transcarbamylase (OTC) for the transport into the matrix space of the mitochondria (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for [rat] rate ornithine transcarbamylase", *Proc. Natl. Acad. Sci. U.S.A.* 80:4258-4262; [J.P] J. P. Kraus *et al.* (1985), "A cDNA clone for the precursor of [rat] rate mitochondrial ornithine transcarbamylase: comparison of [rat] rate and human leader sequences and conservation of catalytic sites", *Nucleic. Acids. Res.* 13:943-952). Basically, the pure signal sequence suffices for the transport into the [aimed] targeted compartment. However, preferable is to select signal sequences which additionally have a cell-specific or compartment-specific peptidase cleavage site. In the most favorable case, [this 'cleavage site']the "cleavage site" is within the signal sequence but can also be attached thereto by additional amino acids to ensure the cleavage of the signal sequence when the [aimed] targeted compartment has been

reached (e.g., the signal sequence of human OTC can be prolonged by ten additional amino acids of the matured OTC). This ensures that the nucleic acid can be separated from the signal peptide in the targeted compartment, so that the action of the nucleic acid fully unfolds. The selected signal sequence is prepared biologically (purification of natural signal sequences or cloning and expression of the signal sequence in a eukaryotic or prokaryotic expression system) but preferably in a chemical-synthetic way.

In order to ensure a linear chemical linkage between nucleic acid and signal peptide, the signal peptide is linked via a linkage agent which is generally linked therewith via amino acids, preferably via amino acids having reactive side groups, preferably via an individual cysteine or lysine at the carboxy-terminal end of the signal peptide. A bifunctional cross-linker serves as a linkage reagent, preferably a heterobifunctional cross-linker which has a second reactive group, preferably an aminoreactive group, in addition to a thiol-reactive group at the signal peptide when a cysteine is used as the linkage site (e.g. m-maleinimidobenzoyl-N-hydroxy-succinimide ester, MBS and its derivatives).

The nucleic acid also has a linkage site which should be compatible with the selected cross-linker. When MBS is used, the oligonucleotide should have an amino function or thiol function. The linkage group of the nucleic acid can be introduced via the chemical synthesis of the oligonucleotide and is generally localized at the 5' end, at the 3' end, but preferably directly at a modified base, e.g., as 5' amino linker (TFA amino linker Amidite^{R}, [1, 6] 1,6-(N-trifluoroacetylamino)-hexyl-β-cyanoethyl-N,N-diisopropyl phosphoramidite, Pharmacia) or a 5' thiol linker (THIOL-C6 Phosphoramidit^{R}, MWG Biotech) at a free 5' hydroxy/phosphate group, as 3' amino linker (3' aminomodifier-C7-CPG-Synthesesäulen^{R}, MWG Biotech) at a free 3' hydroxy/phosphate group, but preferably as amino-modified base analog, preferably amino-modified [deoxyuridine] deoxyuridine (Amino-Modifier-dT^{R}, 5'-dimethoxy-trityl-5[N-(trifluoroacetylaminohexyl)-3-acrylimido]-2'-deoxyuridine, 3'-[2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite, Glen Research) within the sequence. In this case, the reactive group compatible with the cross-linker used is spaced from the 5' end or 3' end of the oligonucleotide or the modified base by at least one C2-spacer unit, but

preferably by a C6-spacer unit. The nucleic acid (oligonucleotide) including a reactive linkage group then comprises at least two nucleotides.

In order to increase the stability of the nucleic acid (oligonucleotide) over cellular and extracellular nucleases, the chemically synthesized nucleic acids can be protected by a sulfurizing reagent (Beaucage-Reagenz^R, MWG- Biotech). The phosphorus diester bonds of the nucleic acid are converted into phosphorus thioate bonds in the chemical synthesis. This oligonucleotide can then be used for the enzymatic amplification of nucleic acids, extended by further linkage reactions with other nucleic acids or used directly.

In order to directly use the [chimerical] chimeric peptide nucleic acid fragment, the nucleic acid (oligonucleotide) should have a secondary structure that can be hybridized, preferably without internal homologies so as to be able to form a linear single-strand structure. This ensures that the nucleic acid (oligonucleotide) of the [chimerical] chimeric peptide-nucleic acid fragment can unfold a biochemical/therapeutic effect without further nucleic acid linkages.

However, for linkage with the signal sequence it is preferred to use nucleic acids (oligonucleotides) which have two further properties:

1. The sequence is preferably partially palindromic, has a blunt 5'-3' end ('blunt end'), an overhanging 3' end ('sticky end'), but [has] especially has an overhanging, phosphorylated 5' end ('sticky end'), especially preferably an overhanging 5' end which comprises 4 nucleotides and has no self-homology (palindromic sequence). As a result, a stable, monomeric secondary structure ('hairpin loop') may form. The overhanging 5' end serves for linking defined nucleic acids, antisense oligonucleotides, but preferably transcribable and replicatable genes.
2. In the apex of the 'loop', the oligonucleotide carries a modified base which carries a grouping reactive with respect to the cross-linker, preferably an amino-modified 2'-deoxythymidine. In this case, the amino function of this modified base enables the linkage reaction between MBS and oligonucleotide.

The [chimerical] chimeric peptide-nucleic acid fragment is suitable for appropriately introducing nucleic acids into cells and cell organelles (e.g. nucleus, chloroplast), particularly for introducing ribonucleic acids (mRNA, 'antisense' oligonucleotides) and deoxyribonucleic acids (complete gene, 'antisense' oligonucleotides). It is especially suitable for the introduction of transcribable and processable genes into mitochondria, but even more suitable for the introduction of replicative, transcription-active and processable linear-cyclic nucleic acids (plasmids).

In a preferred embodiment, a transcribable gene is linked to the nucleic acid, containing the reactive linkage site[,] or to the [chimerical] chimeric peptide-nucleic acid fragment. This is effected preferably by the amplification of a gene, preferably a cloned gene consisting of a mitochondrial promoter, preferably the promoter of the light DNA strand [(0L](O$_L$, nt 490 -nt 369) and the gene to be expressed in a processable form, preferably a mitochondrial gene, preferably a mitochondrial transfer RNA, preferably the mitochondrial [tRNA Leu (UUR)] tRNA$^{Leu(UUR)}$ (nt 3204 - nt 3345) (S. Anderson *et al.* (1981), "Sequence and organization of the human mitochondrial genome", *Nature* 290:457-465). Following the enzymatic amplification of the gene, the linkage to the nucleic acid, containing the reactive linkage site, or to the [chimerical] chimeric peptide-nucleic acid fragment can be effected via a 'blunt end' ligation, but preferably a 'sticky end' ligation. For this purpose, the nucleic acid to be linked has at least one end capable of linkage, which consists preferably of a 5' overhang which comprises 4 nucleotides and has no self-homology (palindromic sequence). If both ends are to be linked with 'hairpin loops', a nucleic acid will preferably be selected which has differing 5' overhangs which comprise 4 nucleotides and have no self-homology. It is especially preferred to use nucleic acids whose 5' ends also have no homology with respect to one another. For the modification of the ends (cyclization) it is then preferred to use two different 'hairpin loops', one being specific (complementary) to the ['left']'left" plasmid end and the other being specific to the 'right' plasmid end of the nucleic acid. In order to increase the stability of the nucleic acid over cellular and extracellular nucleases, the phosphorus diester bonds of the nucleic acid can be substituted with phosphorus thioate bonds and thus be

protected if modified phosphorus thioate nucleotides have been used already in the enzymatic amplification.

A process comprising the following steps is suitable for the production of a [chimerical] chimeric peptide-nucleic acid fragment:

(a) Reaction of a nucleic acid (oligonucleotide), containing a functional linkage group, with a linkage agent.

(b) Reaction of the construct resulting from (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optional extension of the [chimerical] chimeric peptide-nucleic acid fragment resulting from (b) by further DNA or RNA fragments.

In another preferred embodiment, the [chimerical] chimeric peptide-nucleic acid fragment can be produced by the following steps:

(a) Optional extension of the nucleic acid, containing a functional linkage group, by further DNA or RNA fragments.

(b) Reaction of the nucleic acid with functional linkage group or the extended nucleic acid resulting from (a) with a linkage agent.

(c) Reaction of the construct resulting from (b) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL sequence.

In another embodiment which is a linear-cyclic nucleic acid in the form of a plasmid, the selection of the nucleic acid depends on the genetic information which [shall] is to be expressed in which cell and in [which aimed] the particular targeted compartment of the cell. In this connection, nucleic acids which are to be transcribed have to have a suitable promoter. For example, if a gene is to be expressed in the mitochondrial matrix, a mitochondrial promoter can be chosen, preferably the promoter of the light mtDNA strand. The transcription is controlled in other cell compartments (e.g. nucleus, chloroplast) by compartment-specific promoters.

The transcription is usually regulated by what is called transcription regulation sequences, preferably mitochondrial transcription regulation sequences. In general, these

sequences comprise at least binding sites for factors which initiate the transcription (transcription initiation factor) as well as the binding site for the RNA synthesis apparatus. If a transcription is to be initiated in the mitochondria, binding sequences of the mitochondrial transcription factors and of the RNA polymerase, particularly of the mitochondrial transcription factor 1 and the mitochondrial RNA polymerase, will be suitable. In other cell compartments (e.g. nucleus, chloroplast), the transcription can be controlled by compartment-specific transcription-regulation sequences.

In order to be able to regulate the transcription, the plasmid has transcription regulation sequences which are attached preferably in the 3' direction of the transcription initiation site (promoter). For example, if the transcription of a mitochondrial transformation plasmid is to be regulated, the control elements will be suitable for the H-strand and L-strand transcription of the mitochondrial genome[, however preferable]. However in a preferred embodiment, the control elements would be the so-called 'conserved sequence blocks' which terminate the transcription of the L-strand and simultaneously enable the transition [to] of the DNA replication. In order to induce the exclusive transcription of the desired gene (optionally the desired genes in a polycistronic transcription), the transcription is discontinued on a suitable site behind the 3' end of the expressive [gene / genes] gene/genes. This is achieved by the insertion of a suitable transcription-termination site, preferably arranged in the 3' direction to the promoter. For the regulated expression, the binding sequence for a bidirectionally acting transcription-termination factor is especially suitable in this case. For the transcription-termination in the mitochondria, a binding motif of a mitochondrial transcription-termination factor is preferably chosen here. At the same time, the formation of 'antisense RNA' of the head-to-head-linked dimeric plasmids is suppressed by the use of the transcription-termination factor binding sequence.

The selection of transformed cells can be controlled via the expression of a reporter gene. Expressive genes whose expression result in a macroscopic change of the phenotype are especially suitable as reporter or selection gene. A selection is made among genes which produce resistances to antibiotics, for example. In particular, the resistance genes for oligomycin (OLI) or chloramphenicol (CAP) are suitable for the use in a mitochondrial

transformation system. In this connection, the mitochondrial chloramphenicol resistance gene appears to be a particularly suitable selection gene, since CAP-sensitive cell lines already change their phenotype at a portion of about 10[%]% of the 16 S $rRNA^{CAP+}$ gene.

The replication of the nucleic acid can be realized by an initiation site for [the] DNA replication (replication origin). Therefore, the [chimerical] chimeric peptide-nucleic acid fragment in the form of a plasmid has to have at least one replication origin. In this connection, the orientation of the replication origin can be arranged irrespective of the expressive gene (genes), but preferably the replication origin is arranged in the 3' direction of the promoter. A suitable replication origin for a mitochondrial transformation plasmid would be a mitochondrial replication origin. In particular, the origin of replication of the heavy mtDNA strand is suitable in this case. It preferably has at least one ['conserved]"conserved sequence [block']block". The replication can be controlled via what [is] are called regulation sequences for the replication. For this purpose, the plasmid has to have at least one such sequence motif which is preferably arranged in the 3' direction of the promoter and the replication origin. If the replication in the mitochondria is to be regulated, a mitochondrial replication regulation sequence will be especially suitable. It is preferred to use a motif which comprises at least one of the 'termination associated sequences'. In other cell compartments (e.g. nucleus, chloroplast), the replication is initiated at least via one compartment-specific replication origin and controlled via compartment-specific replication origin and controlled via compartment-specific replication regulation sequences.

In order to permit cloning of different genes into the plasmid molecule, the plasmid nucleic acid also has to have a suitable cloning module (multiple cloning site) which has the most widely differing recognition sequences for restriction endonucleases. Here, rare recognition sequences which do not occur on other sites of the plasmid are especially suitable. The cloning module can be incorporated into any site of the transformation plasmid. If the region of the cloning site is to be integrated into the transcription of the selection gene, the insertion of the multiple cloning site in the 3' direction of the promoter and in the 5' direction of the transcription termination site will be suitable. The integration of the multiple cloning site in the 5' direction of the selection gene is especially suitable,

since in this case the use of the selection system is simultaneously accompanied by [a] transcription of the region of the multiple cloning site.

In order to permit the autonomous replication in every [aimed] targeted compartment of a cell when a nucleic acid is used, it has to be ensured that, after the synthesis of the daughter strand, the DNA replication enzymes return to the synthesis starting point again to guarantee the covalent linkage of the 3' end with the 5' end of the newly synthesized daughter strand by corresponding enzymes. For this purpose, a linear nucleic acid plasmid is suitable which can be converted into a cyclic nucleic acid. The plasmid ends can be cyclized via the use of what is called ligation-capable (phosphorylated) [ends] end of nucleic acid. For this purpose, the use of a 'blunt end' nucleic acid or a nucleic acid having [a] overhanging 3' ends, but preferably a nucleic acid having overhanging 5' ends is particularly suitable. In this case, the overhanging ends should comprise at least one nucleotide. However, it is preferred to use overhanging 5' ends which are formed of four nucleotides. They have preferably no self-homology (palindromic sequence) and are also preferably not complementary to one another in order to suppress the formation of dimers in a subsequent nucleic acid linkage.

The cyclization of the prepared plasmid ends is arranged by synthetic oligonucleotides. They have a partial self-homology (partially palindromic sequence) and are thus capable to form what is called 'hairpin loop' structures. The partially palindromic sequence results in the formation of a stable, preferably monomeric secondary structure ('hairpin loop') having a blunt 5'-3' end (blunt end), an overhanging 3' end ('sticky end'), but preferably an overhanging 5' end. These oligonucleotides are especially preferred when they have a phosphorylated 5' end. When synthetic oligonucleotides having 'hairpin loop' structure are used, the linear plasmid DNA can be converted into a linear-cyclic system. The ends of the two oligonucleotides are preferably complementary to one end of the prepared plasmid nucleic acid each. For this purpose, two different 'hairpin loops' are preferably used,[one being specific (complementary) to the 'left' plasmid end,] one being specific (complementary) to the 'right' plasmid end to suppress the dimer formation. At least one of the two 'hairpin loop' oligonucleotides may have at least one modified nucleotide. It guarantees the linkage site to a signal peptide, so that the nucleic acid

transport can be arranged via the protein import route. In the model case, this linkage site (modified nucleotide) is placed at one of the unpaired positions of the 'loop'. A chemically reactive group, particularly an amino or thiol function[,] is especially suitable as linkage site.

In order to prepare the ends of the transformation plasmid [for] of the modification (cyclization), it [has to] must be ensured that the plasmid ends are complementary to the ends of the oligonucleotides ('hairpin loops'). On the one hand, this succeeds by amplifying the plasmid DNA with suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease. In this case, recognition sequences for restriction endonucleases are suitable which do not occur repeatedly in the plasmid sequence. Especially suitable is the use of recognition sequences for restriction endonucleases generating overhanging ends ('sticky ends'), particularly those which produce overhanging 5' ends, preferably outside the own recognition sequence. In this connection, the recognition sequence for the restriction endonuclease *Bsa* I ($GGTCTCN_1N_5$) is especially suitable. On the other hand, the use of a cloned nucleic acid which already has the recognition sequences for a restriction endonuclease, preferably *Bsa* I, is suitable. As a result, the enzymatic amplification can be omitted and the nucleic acid obtained by plasmid preparation/restriction enzyme treatment can be used directly. It is preferred that the cloned nucleic acid already includes the recognition sequence for the restriction endonuclease *Bsa* I at both ends.

Various methods are available for purifying the transformation plasmid. Here, the main objective is to separate the cyclic plasmid molecule from the unreacted educts. The use of DNA-degrading enzymes are proved to be suitable in this connection. In particular, it is recommended to use enzymes which have a 5'-3' or 3'-5' exonuclease activity. Particularly the use of the exonuclease III leads to the complete hydrolysis of unreacted educts while the cyclic plasmid DNA remains intact (no free 5' ends or 3' ends). The reaction products can be purified either via electrophoretic or chromatographic processes but also by precipitation. A selection can be made among different purification processes. On the one hand, the cyclic nucleic acid conjugated with the linkage agent and the signal peptide can be treated with an exonuclease, preferably exonuclease III, and then be purified

via chromatographic[,] electrophoretic purification and precipitation, respectively. On the other hand, the cyclic plasmid DNA can also be treated with an exonuclease, preferably exonuclease III, be purified and subsequently be conjugated with the linkage agent and the signal peptide and be purified via a chromatographic, electrophoretic purification and precipitation, respectively.

The linkage with a signal peptide can be realized by means of modified oligonucleotides. This peptide directs *in vivo* the transformation plasmid into the desired cell compartment. To this end, either the transformation plasmid can first be reacted with the modified oligonucleotide (ligation) and then the conjugation with the linkage agent and the signal peptide can take place or the modified oligonucleotide is first conjugated with the linkage agent and the signal peptide and [can] then be used for the cyclizing the transformation plasmid ends (ligation).

The transformation system (cellular transformation) can overcome the cell membrane by various methods. Here, 'particle gun' system or microinjection are suitable, but electroporation and lipotransfection are preferred. All methods ensure the introduction of the linear-cyclic peptide nucleic acid plasmid into the cytosol of the cell from where the plasmid is directed, to its site of action [(aimed](the targeted compartment) by the conjugated signal peptide.

As compared to the prior art transformation and infection methods, mentioned in the introductory part of the description, this process offers, for the first time, the possibility of appropriately introducing nucleic acids into cells and cell organelles. The selection of the signal sequence can [determined] determine the [aimed] targeted compartment which is to be reached in this case (cytosol, nucleus, mitochondrion, chloroplast, etc.). Along with the compartment-specific and cell-specific introduction behavior, this process distinguishes itself by its universal applicability. Both prokaryotic and eukaryotic cells and cell systems can be treated with the translocation vector. Since a natural transport system of the membranes is used for the appropriate introduction, the treatment of [the] cells or cell organelles with membrane-permeabilizing agents becomes superfluous (e.g. calcium chloride method, see above).

When a replicative and transcription-active nucleic acid is used, the plasmid does not unfold its full size until the first replication cycle has been completed: As a genuine cyclic plasmid (artificial chromosome) it now has the double genetic information (head-to-head linked plasmid dimers). In particular with respect to the use of this system for a somatic gene therapy, this [behaviour] behavior is induced intentionally and of decisive importance, since the genes to be expressed have to compete with the defect genes of the cells. In addition to this highest possible effectiveness, the system distinguishes itself through the fact that it does not have to be integrated into a genome via a recombination step, such as retroviral systems, so as to become replicative. As a result, uncontrollable side-effects (undesired recombination) are already suppressed to the highest possible degree from the start. Therefore, the application of this plasmid system can be expected without great safety risk.

The [present invention is explained particularly by the figures, wherein:

Fig. 1 shows a signal peptide of the ornithine transcarbamylase of rats as well as a DNA sequence suitable for the introduction. Top: signal peptide of the ornithine transcarbamylase of rats (32 amino acids), extended by 10 N-terminal amino acids of the matured protein and an additional cysteine as linkage site. The peptide sequence is shown in the international one-letter code; middle: a partially palindromic DNA sequence suitable f or the introduction and consisting of 39 nucleotides having an amino- modified T at nucleotide position 22; bottom: marked secondary structure of the oligonucleotide having an overhanging 5' end and a modified nucleotide in the vertex of the 'loop'.

Fig. 2 shows the structure of the amino-modified 2'- deoxythymidine, R: nucleic acid residues.

Fig. 3 shows a diagram of the chimerical peptide-nucleic acid fragment, consisting of amino-modified oligonucleotide (39 nucleotides) with marked 'hairpin loop', cross-linker and signal peptide. CL: cross-linker.

Fig. 4 shows the electrophoretic separation of the linkage product resulting from amino-modified oligonucleotide (39 nucleotides), m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) and signal peptide of the ornithine transcarbamylase of rats (42 amino acids, extended by a cysteine at the C terminus).

Fig. 5a shows a flow diagram of the peptide-DNA fusion, cloning, amplification and linkage of the transcribable and processable mitochondrial tRNA gene to be introduced (S. Anderson et al. (1981), "Sequence and organization of the human mitochondrial genome", Nature 290: 457-465). CL: cross-linker (MES); MCS: multiple cloning site of pBluescriptR (Stratagene), mtTF: binding site of the mitochondrial transcription factor; RNA-Pol: binding site of the mitochondrial RNA polymerase; tRNA Leucin: gene of the mitochondrial transfer RNA for leucine (UUR); Sac II, Apa I, Eco RI: sites for restriction endonucleases; the cloned mitochondrial sequences were numbered in accordance with the published sequence of the human mitochondrial genome (S. Anderson et al. (1981), "Sequence and organization of the human mitochondrial genome", Nature 290: 457-465).

Fig. 5b shows the sequence of the cloned tRNALeu (UUR) gene.

Fig. 6a/b shows a presentation of the 32P radiation of the DNA as well as the enzyme activities for adenylate kinase, cytochrome c oxidase and malate dehydrogenase (y axes) in 11 fractions (x axes) of a mitochondria-sucrose gradient density centrifugation. The portion of the particular radiation/enzyme activity, expressed as percentage of the total radiation/enzyme activity which was plotted against the gradient is illustrated. ADK: adenylate kinase; COX: cytochrome c oxidase; MDH: malate dehydrogenase.

Fig. 8 shows the cloning of the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 1). Using the two oligonucleotides (primers 1 and 2), the gene section of nucleotide 15903 to nucleotide 677 was amplified enzymatically from mitochondrial

HeLa DNA (comprises: promoter characterized by the binding sites for the mitochondrial transcription factors and the RNA polymerase; replication origin characterized by what is called 'conserved sequence blocks'; regulation of the DNA replication characterized by the 'TAS' motifs). Since the oligonucleotides contain recognition sequences for the restriction endonucleases Xho I and Pst I, the ends of the amplified nucleic acid can be modified such that they are compatible with a vector arm of pBluescript, on the one hand, and compatible with the hybrid of the oligonucleotides MCS/TTS 1 and 2, on the other hand. In addition to a multiple cloning site, they also comprise a transcription termination sequence which is responsible for the regulated transcription. The ligation product is then transformed into E. coli XL 1. Following the plasmid isolation of insert-carrying E. coli colonies, the nucleic acids were subjected to RFLP and sequence analysis.

Fig. 9 shows the sequence of the oligonucleotides MCS/TTS 1 and 2. The oligonucleotides MCS 1 and 2 were prepared synthetically and comprise recognition sequences for nine different restriction endonucleases as well as a sequence motif which can suppress the transcription bidirectionally. The oligonucleotides are complementary and can thus form a hybrid. The overhanging ends are part of the recognition sequences for the restriction endonucleases Pst I and Bam HI.

Fig. 10 shows the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 1).

Fig. 11 shows the cloning of the reporter gene into the nucleic acid portion of the peptide-nucleic acid plasmid into pBluescript (plasmid 2). Using the two oligonucleotides (primers 3 and 4), the gene section of nucleotide 1562 to nucleotide 3359 was amplified enzymatically from a DNA extract of a human CAP-resistant cell line (comprises: part of the 12 S rRNA gene, tRNAVal gene, 16 S rRNACAP+ gene, tRNALeu gene, part of ND 1 gene). Since the oligonucleotides contain recognition sequences for the restriction endonucleases Hind III and Bcl I, the ends of the amplified nucleic acid can be modified such that they are compatible with the multiple cloning site (MCS) of the peptide-nucleic

acid plasmid (plasmid 1). The ligation product is then transformed in E. coli XL 1 Blue. Following the plasmid isolation of insert-carrying E. coli colonies, the nucleic acids were subjected to RFLP and sequence analysis and are available for the described experiments.

Fig. 12 shows the nucleotide sequence of the nucleic acid portion of the peptide-nucleic acid plasmid including the reporter gene (plasmid 2).

Fig. 13a shows the reaction run of the cyclization of the nucleic acid portion as well as the conjugation of the nucleic acid portion with a signal peptide. The nucleic acid portion of the peptide-nucleic acid plasmid can be obtained via a plasmid preparation or an enzymatic amplification. In both cases, the treatment with the restriction endonuclease Bsa I results in an intermediate product capable of ligation. It can be reacted directly with the monomerized 'hairpin loops'. The reaction product is freed by an exonuclease III treatment from non-specific (non-cyclic) reaction products and products, is purified and conjugated with the signal peptide via a cross-linker. As an alternative, one of the two 'hairpin loops' can first be conjugated with the signal peptide via a cross-linker before the cyclizing ligation reaction is carried out. A purification of the reaction product follows an exonuclease III treatment here as well.

Fig. 13b shows the structure and sequence of the 'hairpin loop' oligonucleotides HP 1 and 2.

Fig. 14 shows the monomerization of oligonucleotide. The synthetic 1 and 2 can be monomerized alkaline denaturation. This standard agarose gel: lane 1, a 'hairpin loop' 'hairpin loops' HP by a thermal or figure shows a molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease Hae III), lane 2: HP 1, synthesis product; lane 3: HP 1, thermally monomerized.

Fig. 15a shows a ligation reaction between the nucleic acid portion of the peptide-nucleic acid plasmid (plasmid 2) and the 'hairpin loops' HP 1 and 2. This figure shows a standard agarose gel: lane 1, cloned nucleic acid portion of the peptide-nucleic acid portion in

pBluescript treated with the restriction endonuclease Bsa I, lane 2: ligation of the reaction products resulting from lane 1 with the 'hairpin loops' HP 1 and 2; lane 3, treatment of the reaction products resulting from lane 2 with exonuclease III; lane 4, molecular weight standard (λDNA treated with the restriction endonucleases Hind III and Eco RI).

Fig. 15b shows the examination of the purified ligation product by a Mae III-RFLP analysis. This figure illustrates a standard agarose gel: lane 1, enzymatically amplified nucleic acid portion following a Mae III treatment; lane 2: purified ligation product of the enzymatically amplified nucleic acid portion following a Mae III treatment; lane 3: purified product of the plasmid DNA ligation following a Mae III treatment; lane 4, molecular weight standard (ΦX 174 RF DNA treated with the restriction endonuclease Hae III).

Fig. 16 shows the transcription and replication of the peptide-nucleic acid plasmid. This figure illustrates a standard agarose gel: lane 1, molecular weight standard (λDNA treated with the restriction endonucleases Hind III and Eco RI); lane 2, untreated peptide-nucleic acid plasmid; lane 3: in vitro-obtained transcription products of the peptide-nucleic acid plasmid; lane 4: in vitro-obtained replication and transcription products of the peptide-nucleic acid plasmid; lane 5, in vivo-obtained replication and transcription products of the peptide nucleic acid plasmid; lane 6, untreated peptide-nucleic acid plasmid.

The present invention is now explained by way of the below examples which, however, shall not at all restrict the invention.

Example 1:

Introduction of a chimerical peptide-nucleic acid fraqment into the mitochondria] below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described

herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLES

A. Example 1: Introduction of a Chimeric Peptide-Nucleic Acid Fragment Into the Mitochondria

The overcoming of the mitochondrial double membrane system with a DNA translocation vector was studied to prove that nucleic acids can be transported appropriately across membranes by the above-described process. For this purpose, the mitochondrial signal sequence of the ornithine transcarbamylase (A.L. Horwich *et al.* (1983), "Molecular cloning of the cDNA coding for rat ornithine transcarbamylase", *Proc. Natl. Aca. Sci. U.S.A.* 80:4258-4262) (enzyme of urea cycle, naturally localized in the matrix of the mitochondria) was chemically prepared and purified. The original sequence was extended by a cysteine at the C terminus as reactive group for the subsequent linkage with the DNA (see fig. 1). This ensured that the heterobifunctional cross-linker (MBS) can only be linked with the thiol group of the only cysteine. A DNA oligonucleotide (39 nucleotides) were chosen as linkage partner. It distinguishes itself by two special features:

1. The sequence is partially palindromic and has [an] and overhanging, phosphorylated 5' end (see fig. 1). As a result, what is called a 'hairpin loop' can form. The overhanging 5' end serves for ligating to this oligonucleotide defined nucleic acids which can then be imported into the mitochondria.
2. The oligonucleotide carries a modified base in the vertex of the ['1oop']'1oop' (see fig. 1). In this case, an amino-modified 2'-deoxythymidine is concerned (see fig. 2). Here, the amino function of the modified bases in this connection enables the linkage reaction between MBS and oligonucleotide.

The three reaction partners (oligonucleotide, MBS and peptide) are linked in individual reaction steps. Firstly, the oligonucleotide (50 pmoles) is reacted in a buffer (100 [μl]µl; 50 mM potassium phosphate, pH 7.6) with MBS (10 nmoles dissolved in DMSO) (reaction time: 60 min.; reaction temperature: [20°C)] 20°). Unreacted MBS is separated

via a Nick-spin column^{R}, (Sephadex G 50, Pharmacia) which was [equilibrated] equilibriated with 50 mM of potassium phosphate (pH 6.0). The eluate contains the desired [reaction product and is reacted in another] reaction step with the peptide (2.5 nmoles) (reaction time: 60 min.; reaction temperature 20°C). The reaction was stopped by the addition of dithiothreitol (2 mM). The linkage product (chimera, see fig. 3) was separated via a preparative gel electrophoresis of unreacted educts and isolated from the gel by electroelution (see fig. 4). Differing nucleic acids can now be linked by simple ligation to the overhanging 5' end of the oligonucleotide.

A 283 bp long double-stranded DNA (dsDNA) was amplified via an enzymatic reaction (PCR) in the below experiment. For this purpose, a DNA fragment cloned [into] in to pBluescript^{R} (Stratagene) served as template DNA, which fragment in addition to the human mitochondrial promoter of the light strand (P_L, nt 902 - nt 369) included the gene for the mitochondrial transfer RNA leucine [(tRNA Leuc (UUR)](tRNA$^{Leuc(UUR)}$, nt 3204 - nt 4126) (see fig. 5). Two oligonucleotides served as amplification primers, primer 1 having a non-complementary 5' end (see fig. 5). The dsDNA was modified by the 3'-5' exonuclease activity of the T4 DNA polymerase (incubation in the presence of 1 mM dGTP) which can produce overhanging 5' ends under conditions with which a person skilled in the art is familiar (C. Aslanidis *et al.* (1990), "Ligation-independent cloning of PCR products (LIC-PCR)", *Nucleic. Acids[.] Res.* 18: 6069-6074).

Together with the previously conjugated peptide-MBS oligonucleotide the PCR-amplified DNA could be joined using the T4 DNA ligase. In order to be able to easily detect the linkage partners after the introduction into the mitochondria, the free 5'-OH group of the ligated DNA was phosphorylated radioactively by an enzymatic reaction (A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid, I. Phosphorylation at 5'-hydroxyl termini", *J. Biol. Chem.* 241: 2923-2932; A. Novogrodsky *et al.* (1966), "The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid. [II] I. Further properties of the 5'-[hydroxyl] hydroxy polynucleotide kinase", *J. Biol. Chem.* 241: 2933-2943).

A fresh rat liver was comminuted for the isolation of mitochondria, suspended in 25 mM HEPES, 250 mM saccharose, 2 mM EDTA, 52 [μM]μM BSA and homogenized in a

glass homogenizer (50 ml). Cell membranes, cellular debris and nuclei were centrifuged [off at] of fat 3000 g and the supernatant was prepared for another centrifugation. For this purpose, the supernatant was placed in cooled centrifuge cups and centrifuged at 8000 g. The [isolated] isolation mitochondria were resuspended in 200 ml of the same buffer and centrifuged again at 8000 g. The purified mitochondria pellet was resuspended in an equal volume of the same buffer and energized by the addition of 25 mM succinate, 25 mM pyruvate and 15 mM malate. The protein content of the suspension was determined by a Bradford Testkit^{R} (Pierce). 200 [μg]µg of mitochondrial protein (energized mitochondria) were incubated together with 10 pmoles of the chimera at 37°C for 60 min. (0.6 M sorbitol, 10 mM potassium phosphate pH 7.4, 1 mM ATP, 2 mM [MgC12, 1%] $MgCL_2$, 1 % BSA). The mitochondria were reisolated by centrifugation at 8000 g, resuspended in 0.6 M sorbitol, 10 mM potassium phosphate pH 7.4, 2 mM [MgCl2, 1%] $MgCL_2$, 1 % BSA, 10 U/ml DNAse I and incubated at 37°C for 30 min. This washing step was repeated twice to remove non-specifically adhering molecules. For proving that the chimera is associated with the mitochondria, the re-isolated mitochondria were purified via sucrose gradient density centrifugation. The individual fractions of the gradient were analyzed to localize the chimera and the mitochondria. The adenylate kinase which determines cytochrome-c oxidate and malate dehydrogenase activity was used as marker for the mitochondria, while the chimera could be identified via the $^{[32P]}\,^{32}p$ radiation measurement (see fig. 6). An analog experiment for determining the non-specific DNA introduction was carried out with the same DNA which was not linked with the signal peptide (see fig. 6). It was derived from the measurements that 65% of the chimera used segregated specifically with the mitochondria, whereas the non-specific DNA incorporation was less than 5% of the DNA used. In order to show that the chimera is not only associated with the surface of the mitochondria (membrane, import receptor), the re-isolated mitochondria were not fractioned into the three compartments of outer mitochondria membrane/intermembranous space, inner mitochondrial membrane and matrix space. For this purpose, the mitochondria were incubated with digitonin (final concentration: 1.2[%]% w/v digitonin) and the resulting mitoplasts were separated via a sucrose gradient density centrifugation, collected in fractions and the activities of marker enzymes (adenylate kinase: intermembranous space,

cytochrome c oxidase: inner mitochondrial membrane; malate dehydrogenase: matrix space) were determined according to Schnaitman and Grennawalt (C. Schnaitman *et al.* (1968), "Enzymatic properties of the inner and outer membranes of rat liver mitochondria", *J. Cell Biol.* 38: 158-175; C. Schnaitman *et al.* (1967), "The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria", *J. Cell Biol.* 32: 719-735) (see fig. 7). An analog experiment for determining the non-specific DNA incorporation was carried out with the same DNA which was not linked with the signal peptide (see fig. 7). It was derived from the measurements that 45% of the chimera are associated with the mitoplasts, whereas the non-specifically adhering DNA could be assessed to be less than 3%. The isolated mitoplasts (loss of the outer membrane and the intermembranous space) were lyzed by [LubrolR] Lubron^{R} (0.16 mg/mg protein; ICN) and separated into the compartments of inner mitochondrial membrane (pellet) and matrix space (supernatant) by ultracentrifugation at 144,000 g. The compartments were assigned via the measurement of the activities of the cytochrome c oxidase (inner mitochondrial membrane) and the malate dehydrogenase (matrix space). The chimera was measured via the detection of the $^{[32P]}$P radiation in the scintillation counter and the result was 75% segregation with the matrix of the mitochondria, while 25% of the chimera remained associated with the inner membrane of the mitochondria (incomplete translocation).

[Example 2

Incorporation of a replicative and transcription-active chimerical peptide-nucleic acid fragment (plasmid) into the mitochondria of living cells]

B. Example 2: Incorporation of a Replicative and Transcription-Active Chimeric Peptide-Nucleic Acid Fragment (Plasmid) Into the Mitochondria of Living Cells

In order to prove that a linear peptide-nucleic acid plasmid having cyclic ends ('hairpin loops') can overcome membranes *in vivo* via the protein import route and can be transcribed and replicated in spite of the chemical linkage with a signal peptide, the transcription and replication behavior were [studied] studies after the transfection of cells and the import into the matrix of the mitochondria. For this purpose, the signal peptide of

the mitochondrial ornithine transcarbamylase was prepared synthetically, purified and linked with a nucleic acid plasmid.

Precondition for the examination of the correct transcription and replication behavior is the physical structure of the plasmid: for the experiment described below, a 3232 bp long double-stranded vector DNA (dsDNA) was cloned into pBluescript^R (Stratagene). For this purpose, the region of the mitochondrial genome was amplified via two modified oligonucleotides [(primer](primer 1, hybridized with the nucleotides 15903-15924 of the human mtDNA, includes at the 5' end an extension by the sequence TGTAGctgcag (SEQ ID NO:17) for the incorporation of a *Pst* I site; primer 2, hybridized with the nucleotides 677-657 of the human mtDNA, includes at the 5' end an extension by the sequence TTGCATGctcgagGGTCTCAGGG (SEQ ID NO:18) for the incorporation of [an] the *Xho* I site), which comprised the promoter of the light DNA strand, [the origin of the mtDNA replication of the heavy strand,] the regulation motifs for the transcription (CSBs, 'conserved sequence blocks') as well as the regulation site for the DNA replication ('TAS', termination associated sequences, (D.C. Wallace (1989), "Report of the committee on human mitochondrial DNA", Cytogenet. *Cell Genet.* 51:612-621) (see fig. 8). A multiple cloning site [was inserted behind this fragment (3' direction), which is to permit an easy linkage with a gene to be expressed. The multiple cloning site](MCS/TTS) was produced via a chemical synthesis of two complementary oligonucleotides (MCS/TTS 1 and 2) which contain the recognition sequences for various restriction endonucleases (see fig. 9). Under conditions with which a person skilled [in] the art is familiar, the two oligonucleotides form hybrids which, after the phosphorylation with T4 DNA polynucleotide kinase, can be used for the ligation. In this connection, the hybrids distinguish themselves by 5' and 3' single-stranded-overhanging ends which are complementary to a *Pst* I, on the one hand, and are complementary to a *Bam* HI site, on the other hand (see fig. 9). Together with the multiple cloning site, the synthetic oligonucleotides MCS/TTS 1 and 2 also comprise a bidirectional mitochondrial transcription termination sequence (see fig. 9). It is arranged in the 3' direction of the MCS and ensures that the transcription on this site is discontinued thus correctly forming terminated transcripts forming. This sequence motif also ensures that in the cyclic plasmid

system no 'antisense RNA' is expressed. The ligation reaction between pBluescript, PCR-amplified fragment and the MCS/TTS hybrids took place in a stoichiometry of 1:2:2 under conditions with which a person skilled in the art is familiar. After the transformation, several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to dideoxy sequencing (fig. 10) under conditions with which a person skilled in the art is familiar.

For the experimental examination of the replication and transcription, what is called a reporter gene was inserted in the multiple cloning site. The chloramphenicol-resistant human mitochondrial 16 S ribosomal RNA was chosen as the reporter gene. It distinguishes itself from the naturally occurring ribosomal RNA only by a modified nucleotide (polymorphism). By means of the polymerase chain reaction, a fragment having two modified [oligonucleotides] ologonucleotides (primer 3, hybridized with the nucleotides 1562-1581 of the mitochondrial DNA, extended at the 5' end [by] of the sequence CCTCTaagctt (SEQ ID NO:19) for the incorporation of a *Hind* III site; primer 4, hybridized with the nucleotides 3359-3340, extended at the 5' end [by] of the sequence GCATTactagt (SEQ ID NO:20) for the incorporation of a *[Bcl] Bcl* I site) was amplified from a DNA extract of chloramphenicol-resistant HeLa cells under conditions with which a person skilled in the art is familiar. In order to ensure a correct processing of the subsequent transcript, the amplification product included the two flanking tRNA genes ($tRNA^{Val}$ and $tRNA^{Leu}$). The amplified DNA was treated with the restriction endonucleases *Hind* [III] II and *Bcl* I, purified by precipitation and used with the pBluescript plasmid 1 treated with *Hind* III and *Bcl* I (see figs. 8, 9 and 10) in a stoichiometry of 1:1 in a ligation reaction under conditions with which a person skilled in the art is familiar. The cloning strategy is illustrated in fig. 11.

Several E. coli colonies (clones) could be isolated and characterized. For this purpose, the corresponding plasmid DNA was subjected to a dideoxy sequencing under conditions with which a person skilled in the art is familiar (see fig. 12). In order to prepare the cloned DNA for the application to cell cultures and mitochondria, the cloning insert (mitochondrial transformation plasmid) was separated by the use of the restriction endonuclease *Bsa* I from the pBluescript vector under conditions with which a person

skilled in the art is familiar. Alternatively, the insert DNA could be amplified via two oligonucleotides (primers 2 and 5; nucleotide sequence of primer 5: GATCCGGTCTCATTTTATGCG [)](SEQ ID NO:21)) by the polymerase chain reaction. The use of S-dNTPs permitted the production of 'thionated' DNA which is stabilized over cellular nucleases. In both cases, the subsequent use of the restriction endonuclease *Bsa* I resulted in two different 5' overhangs. They are complementary to the 'hairpin loops' used in order to achieve a cyclization of the linear nucleic acid (see figs. 13a and b). The oligonucleotides are produced via chemical synthesis. As a result, they do not have phosphorylated 5' ends and have to be phosphorylated by a kinase reaction under conditions with which a person skilled in the art is familiar (in order to be able to subsequently examine the cellular transformation, [[ν]γ -^{32}P]-ATP was partially used in this reaction as a substrate to radioactively label the plasmid). A majority of the 'hairpin [loop']loop' structure of the oligonucleotides forms spontaneously, since the palindromic sequence can hybridize with itself. However, dimers of the 'hairpin loops' can also be converted into monomers by denaturing them in the greatest possible volume (<0.1 [μM)]μM) at 93°C for at least 5 min. and fixing them immediately in a solid matrix by freezing. Then, the oligonucleotides are slowly thawed at 4°C and then 99% thereof are available in the desired monomeric 'hairpin loop' structure (see fig. 14).

The plasmid DNA was cyclized together with the two monomerized 'hairpin loops' (HP 1 and 2) in a reaction batch. In this case, the molar ratio [of] plasmid DNA to the two 'hairpin loops' was 1:100:100 (plasmid:HP1:HP2). By using the T4 DNA ligase, the individual reactants could be combined under conditions with which a person skilled [in] the art is familiar (see fig. 15). The ligation products were purified by a treatment with exonuclease III (reaction conditions: 37°C, [60] 50 min.). While nucleic acids having free 3' ends are decomposed by the nuclease, the plasmid DNA linked with the two 'hairpin loops' remains stable over the 3'-5' exonuclease activity of the enzyme. The only reaction product (see fig. [15a)] 15a) was separated via a preparative agarose gel electrophoresis and purified by an electroelution or by using QIAquick (Qiagen) in accordance with the manufacturer's recommendation.

The ligation product was examined via an RFLP analysis (restriction fragment length polymorphism). For this purpose, the ligated and purified plasmid DNA was treated with the restriction endonuclease *Mae* III under conditions with which a person skilled in the art is familiar. The DNA had five cleavage sites, so that fragments of differing [sizes] dizes form which can be analyzed via an agarose gel (4%). Fig. [15b] 15b shows by way of example the *Mae* III cleavage pattern that is obtained after the ligation of the plasmid DNA with the two 'hairpin loops'. In this case, the DNA bands marked by the arrow tips represent the left and right end of the amplified (lane 1) and the linear-cyclic (lanes 2 and 3) mitochondrial plasmids.

For the conjugation of the circularized plasmid with the synthetic signal peptide of the rat [ornithine] orninthine transcarbamylase [(H2NMLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQVQ-LKPRDLC-COOH)](H2 N-MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQVQ-LKPRDLC-COOH (SEQ ID NO:22)), the nucleic acid was incubated with 20 times a molar excess of m-maleimidobenzoyl-N-hydroxysuccinimide ester (linkage agent) at 20°C for 60 min. (incubation medium: 50 mM potassium phosphate pH 7.8). The excess linkage agent was separated by a 'nick spin column' (Pharmacia-LKB) under conditions with which a person skilled in the art is familiar. The 'activated' nucleic acid was conjugated by reacting the nucleic acid with 50 times the molar excess of the signal peptide at 20°C (incubation medium: 50 mM potassium phosphate pH [6.8)] 7.8). The reaction was stopped by the addition of 1 mM dithiothreitol after 45 min. and the conjugate was available for the experiments to come.

In order to be able to show the *in vivo* usability of the peptide-nucleic acid plasmid, the plasmid had to be incorporated into eukaryotic cells. For this purpose, a chloramphenicol-sensitive B lymphocyte or fibroblast cell culture was transfected via a lipotransfection with the peptide-nucleic acid plasmid [: 1 μg of the radioactively labeled peptide-nucleic acid plasmid](the labeling was introduced [as 32P] at ^{32}p labeling during the kinase reaction of the 'hairpin loop' (HP1)) was pre-incubated together with 2-6 [μl LipofectAmineR (Gibco-BRL) in 200 μl]µl serum-free Optimem^{R} (Gibco-BRL) (20°C, 15 min.). During the incubation the polycationic lipid of the [LipfectAmineR] LipofectAmine^{R}

reagent DOSPA (2,3-dioleyloxy-N-[2-(sperminecarboxamido)-ethyl]-N,N-dimethyl-1-propaneaminiumtrifluoroacetate) forms [unilamellar] unillamellar liposomes with the aid of the neutral lipid DOPE [(dioleoylphosphatidyiethanolamine)](dioleoylphosphatidylethanolamine), which can complex the DNA. Then, the reaction batch was added to the prepared cells, adjusted to a density of about $2.5*10^6$ cells per 0.8 ml (35 mm culture dishes, 4 h, 37°C, CO_2 incubator). The transfection medium was then replaced by 5 ml of DMEM medium (Gibco-BRL) previously supplemented by 10% fetal calf serum and 100 [μg/ml]µg/ml chloramphenicol. The transformation efficiency was determined by the measurement of the ^{32}P radiation of the construct. As a rule, a cellular incorporation rate of 80-85% [was measured. This means that 80-85% of the chimerical] of the chimeric construct were associated with the transformed cells and 15-20% of the [chimerical] chimeric peptide-DNA plasmid remained in the supernatant of the transfection reaction.

After about 21-28 days, chloramphenicol-resistant colonies formed in the transformed cells. Under conditions with which a person skilled in the art is familiar, the resistant cells were isolated and multiplied. Under conditions with which a person skilled in the art is familiar, sufficient DNA could be obtained from about $1*10^5$ cells to classify the genotypes. For this purpose, the isolated DNA was separated via agarose gel electrophoresis and transmitted to a nylon membrane (Southern blot). The nucleic acids were detected by hybridization using a specific, radioactively labeled probe (see fig. 16). In addition to the introduced circularized 'linear' vector (lanes 2 and 6) [a] an 'in vitro' transcription (lane 3), an 'in vitro' replication (lane 4), as well as the intermediates obtained 'in vivo' (isolated nucleic acids of a transformed clone) are shown in this illustration. While the three smaller bands can be produced in vitro by incubating the circularized vector with the four nucleoside triphosphates (RNA) and a mitochondrial enzyme extract (lane 3), the formation of a dimer, circular plasmid (greatest band in lane 4) is observed in the further addition of the deoxynucleoside triphosphates to the reaction batch: an identical image yields the analysis of the nucleic acids which can be obtained from transformed cell colonies (lane 5). The fact that the greatest DNA band in lanes 4 and 5 is actually the

dimeric and thus replicated mitochondrial plasmid, could be confirmed by sequence analysis.

A lipotransfection batch where the non-conjugated plasmid not linked with the signal peptide was used, served as a control experiment. As expected, this plasmid was not incorporated into the mitochondria of the transfected cells and thus did not result in the formation of chloramphenicol-resistant cells. These cells stopped growth after 10 days and decayed within the following 8 to 10 days completely.

[Claims]

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

[1)]

CLAIMS

WHAT IS CLAIMED:

1. A chimerical peptide-nucleic acid fragment comprising:

(a) a cell-specific, compartment-specific or membrane-specific signal peptide, with the exception of a KDEL signal sequence,

(b) a linkage agent,

(c) a nucleic acid (oligonucleotide),

the signal peptide being linked via the linkage agent which via amino acids at the carboxy-terminal end of the signal peptide is linked therewith so as to ensure the appropriate nucleic acid introduction into cell organelles and cells.

2[)]. The chimerical peptide-nucleic acid fragment according to claim 1, characterized in that the nucleic acid consists of at least two bases.

3[)]. The chimerical peptide-nucleic acid fragment according to [claim 1 or] any one of claims 1 to 2, characterized in that the nucleic acid has a [hybridizable] secondary structure.

4[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 3, characterized in that the nucleic acid has a palindromic sequence.

5[)]. The chimerical peptide-nucleic acid fragment according to claim 4, characterized in that the nucleic acid may form a ['hairpin loop']"hairpin loop".

6[)]. The chimerical peptide-nucleic acid fragment according to claim 5, characterized in that the nucleic acid may hybridize with itself and may form an overhanging 3' end or 5' end ('sticky end').

7[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 6, characterized in that the nucleic acid is a ribonucleic acid, preferably a deoxyribonucleic acid.

8[]. The chimerical peptide-nucleic acid fragment according to claim 7, characterized in that the nucleic acid has chemically modified ['phosphorus]'phosphorous thioate' linkages.

9[]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 8, characterized in that the nucleic acid carries a reactive linkage group.

10[]. The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains an amino function when the linkage agent contains an amino-reactive grouping.

11[]. The chimerical peptide-nucleic acid fragment according to claim 9, characterized in that the reactive linkage group contains a thiol function when the linkage agent contains a thiol-reactive grouping.

12[]. The chimerical peptide-nucleic acid fragment according to claim 10 or 11, characterized in that the linkage grouping present is bound to the nucleic acid via at least one C2 spacer, but preferably one C6 spacer.

13[]. The chimerical peptide-nucleic acid fragment according to claim 12, characterized in that the linkage grouping is localized at the 3' hydroxy/phosphate terminus or at the 5' hydroxy/phosphate terminus of the nucleic acid, but preferably at the base.

14[]. The chimerical peptide-nucleic acid fragment according to any one of claims 10 to 13, characterized in that defined nucleic acids, antisense oligonucleotides, messenger RNAs or transcribable and/or replicatable genes are linked with the 5' end and/or 3' end.

15[]. The chimerical peptide-nucleic acid fragment according to claim 14, characterized in that the nucleic acid to be linked contains chemically modified 'phosphorus thioate' linkages.

16[)]. The chimerical peptide-nucleic acid fragment according to claim 14 [or] to 15, characterized in that the gene [to] be linked contains a [promoter] promotor, preferably a mitochondrial promoter.

17[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 16, characterized in that the signal peptide has a reactive amino acid at the carboxy-terminal end, preferably a lysine or cysteine, when the linkage agent contains an amino-reactive or thiol-reactive grouping.

18[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 17, characterized in that the signal peptide carries a cell-specific, compartment-specific or membrane-specific recognition signal.

19[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 18, characterized in that the signal peptide has a cell-specific, compartment-specific or membrane-specific peptidase cleavage site.

20[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 19, characterized in that the peptide consists of the compartment-specific cleavable signal peptide of the human mitochondrial ornithine transcarbamylase, extended by an artificial cysteine at the C terminus.

21[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 20, characterized in that the linkage agent is a bifunctional, preferably heterobifunctional[,] cross-linker.

22[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 21, characterized in that the linkage agent contains thiol-reactive and/or amino-reactive groupings when the signal peptide and the nucleic acid carry thiol and/or amino groups as linkage sites.

23[]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 22, characterized in that the linkage agent is m-maleimido-benzoyl-N-hydroxy-succinimide ester or a derivative thereof.

24[]. The chimerical peptide-nucleic acid fragment according to any one of claims 1 to 23, characterized in that the molecule can overcome membranes with and without membrane potential by utilizing natural transport mechanisms.

25[]. The chimerical peptide-nucleic acid fragment in the form of a linear-cyclic plasmid, characterized in that the plasmid comprises at least one replication origin and that both ends of the nucleic acid portion are cyclized, at least one cyclic end having a modified nucleotide which via a linkage agent can be [linked] liked with a cell-specific, compartment-specific or membrane-specific signal peptide.

26[]. The chimerical peptide-nucleic acid fragment according to claim 25, characterized in that the nucleic acid portion further comprises at least one promoter, preferably a mitochondrial promoter, especially preferably the mitochondrial promoter of the light strand.

27[]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 and 26, characterized in that the nucleic acid portion further comprises transcription-regulatory sequences, preferably mitochondrial transcription-regulatory sequences.

28[]. The chimerical peptide-nucleic acid fragment according to any one of [claims] Claims 25-27, characterized in that the transcription-regulatory sequences have at least one binding site of a transcription initiation factor.

29[]. The chimerical peptide-nucleic acid fragment according to any one of [claims] Claims 25 to 28, characterized in that the transcription-regulatory sequences have at least

one binding site for the RNA synthesis apparatus, preferably the binding site for the mitochondrial []transcription factor 1 and the mitochondrial RNA polymerase.

30[]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 29, characterized in that the transcription-regulatory sequences are arranged in the 3' direction of the promoter.

31[]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 30, characterized in that the transcription is regulated by elements of the mitochondrial H-strand and L-strand transcription control.

32[]. The chimerical peptide-nucleic acid fragment according to claim 31, characterized in that what is called 'conserved-sequence-blocks' of L-strand transcription act as transcription control elements.

33[]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 32, characterized in that the plasmid further comprises at least one transcription termination site.

34[]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 33, characterized in that the transcription termination site has a binding sequence of a mitochondrial transcription termination factor.

35[]. The chimerical peptide-nucleic acid fragment according to claim 34, characterized in that the transcription termination site has the binding sequence of a preferably bidirectionally acting transcription termination factor.

36[]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 35, characterized in that the replication origin is a mitochondrial replication origin,

preferably the replication origin of the heavy mtDNA strand having at least one ['conserved]'conserved sequence block'.

37[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 36, characterized in that the plasmid further comprises at least one regulatory sequence for the replication.

38[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 37, characterized in that the regulatory sequence for the replication is a mitochondrial sequence motif.

39[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 38, characterized in that the plasmid further comprises a selection gene, preferably an antibiotic-resistance gene, preferably the oligomycin - or chloramphenicol - resistance gene.

40[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 39, characterized in that the plasmid further contains a multiple cloning site which permits the expression of ['foreign]'foreign genes'.

41[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 40, characterized in that the multiple cloning site comprises recognition sequences for restriction endonucleases which do preferably not occur in another site of the plasmid.

42[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 41, characterized in that the multiple cloning site is arranged in the 3' direction of the promoter and in the 5' direction of the transcription termination site.

43[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 42, characterized in that the multiple cloning site is arranged in the 5' direction of the selection gene.

44[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 43, characterized in that the nucleic acid fragment has (phosphorylated) ends capable of [ligation] litigation.

45[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 44, characterized in that the nucleic acid fragment has ['blunt]'blunt ends' or overhanging 3' ends, preferably overhanging 5' ends.

46[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 45, characterized in that the nucleic acid fragment has 4 nucleotides comprising 5' overhangs which do not have a self-homology (palindromic sequence) and are not complementary to one another either.

47[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 46, characterized in that the ends of the nucleic acid fragment are cyclized via synthetic [oligonucleot ides.] oligonucleotides.

[48)]

48. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 47, characterized in that the overhanging 5' ends of the two oligonucleotides are complementary to one differing end of the nucleic acid each.

49[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 48, characterized in that two differing ['hairpin]'hairpin loops' are used for the cyclization, one being specific (complementary) to the ['left']'left' plasmid end and the other being specific to the ['right']'right' plasmid end of the nucleic acid.

50[)]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 49, characterized in that the modified nucleotide is localized preferably within the ['loop']'loop'.

51[]. The chimerical peptide-nucleic acid fragment according to any one of claims 25 to 50, characterized in that the plasmid DNA is amplified enzymatically by suitable oligonucleotides which have at least one recognition sequence for a restriction endonuclease which occurs preferably in non-repeated fashion in the plasmid sequence.

52[]. The chimerical peptide-nucleic acid fragment according to claim 51, characterized in that the restriction endonuclease to be used generated overhanging ends, preferably 5' overhanging ends, the cleavage site being localized preferably outside the recognition sequence.

53[]. The chimerical peptide-nucleic acid fragment according to claim 51 or 52, characterized in that the restriction endonuclease is [Bsa I] *BsaI*.

54[]. A process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following [steps] stages:

(a) reaction of a nucleic acid (oligonucleotide) containing a functional linkage group having a linkage agent,

(b) reaction of the construct of (a) with amino acids at the carboxy-terminal end of a peptide, containing a signal sequence, with the exception of a KDEL signal sequence, and

(c) optionally extension of the chimerical peptide-nucleic acid fragment of (b) by further DNA or RNA fragments.

55[]. The process according to claim 54, characterized in that the DNA in step (c) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine [(tRNALeu(UUR)).](tRNALeu^{UUR}).

[56)]

56. The process for the production of a chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53, characterized by the following steps:

(a) optional extension of the nucleic acid containing a functional linkage group by further DNA or RNA fragments,

(b) reaction of the nucleic acid with functional linkage group or the extended nucleic acid of (a) with a linkage agent,

(c) reaction of the construct of (b) with amino acids at the carboxy-terminal end of a peptide containing a signal sequence, with the exception of a KDEL signal sequence.

57[)]. The process according to claim 56, characterized in that the DNA in step (a) is a PCR-amplified DNA fragment containing the human mitochondrial promoter of the light strand (P_L) as well as the gene for the mitochondrial transfer RNA leucine [(tRNALeu(UUR)](tRNALeu$^{UUR)}$).

58[)]. Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 53 for the appropriate nucleic acid introduction into cell organelles and cells, characterized by reacting the fragment with cells or pretreated cell compartments.

59[)]. Use according to claim 58, characterized in that the pretreated cell compartments are energized mitochondria.

60[)]. Use of the chimerical peptide-nucleic acid fragment according to any one of claims 1 to 59 for the introduction into eukaryotic cells.

61[)]. Use of a chimerical peptide-nucleic acid fragment according to claim 60, characterized by employing the ['particle]'particle gun' system, electroporation, microinjection or lipotransfection for the introduction into eukaryotic cells.

ABSTRACT

This invention relates to a chimeric peptide-nucleic acid fragment, the process for producing the same and its use for appropriately introducing nucleic acids into cell organelles and cells.